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iology of the Mycobacterioses

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE Center for Disease Control

Bacteriology of the Mycobacterioses

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"But in the future the battle against this plague of mankind [tuberculosis] will not just be concerned with an uncertain something, but with a tangible parasite, about whose characteristics a great deal is known and can be further explored."
R. Koch, 1882.

U. S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
Public Health Service
Center for Disease Control
Bureau of Laboratories
Bacteriology Division
Mycobacteriology Branch
Atlanta, Georgia 30333
MARCH 1976

HEW Publication No. (CDC) 76-8316

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(This work was done in partial fulfillment of an agreement between the World Health Organization and the Mycobacteriology Branch, Center for Disease Control, U.S. Department of Health, Education, and Welfare, Atlanta, Georgia.)

For sale by the Superintendent of Documents, U.S. Government Printing Office
Washington, D.C. 20402

ACKNOWLEDGEMENTS

The author wishes to thank his co-workers in the Mycobacteriology Branch, Center for Disease Control, for their help, suggestions, and encouragement, and Dr. P. Q. Edwards, Tuberculosis Division, CDC, and Dr. G. Kubica, Laboratory Training and Consultation Division, CDC, for their critical review of the manuscript and helpful suggestions. He also wishes to thank Dr. A. Balows, for his support and encouragement in this endeavor. Special thanks go to numerous colleagues and the publishers of scientific journals who gave us permission to reproduce from their work very valuable information.

I also wish to thank Mrs. Frances Carithers, Mycobacterial Immunology Section, who did the typing of the manuscript, Janet Stewart Fulmer, Publications Graphics, who did the graphics, and Miss Edwina Davis, Publications Activities, who edited the manuscript.

Preface

Medical mycobacteriology encompasses a few strictly parasitic and pathogenic mycobacteria, some potentially pathogenic ones, and a larger number that can be isolated from human secretions, but which are not known to cause disease. In the not too distant past, the main medical problem in this field was to recognize tuberculosis and its etiologic agent (*Mycobacterium tuberculosis*). Medical students and bacteriologists were taught, and trained, to differentiate five types or varieties of the tubercle bacilli (the human, the bovine, the avian, the murine, and the cold-blooded animal types). These types were distinguished on the basis of their differential pathogenicity in experimental animals. Because in most cases tuberculosis in humans is caused by the human type, the main criterion in the diagnosis of tuberculosis was the demonstration of progressive tuberculosis in guinea pigs inoculated with the pathological specimens from the patient. Inoculation in rabbits differentiated the human and bovine types.

The microscopic observation of bacilli exhibiting the unique property of acid fastness could be accepted as of diagnostic significance in specimens examined for pulmonary tuberculosis or in exudates drawn from closed lesions, but was not generally accepted in the case of specimens examined for tuberculosis of the kidneys, since it was recognized very early that saprophytic mycobacteria could be observed in urine sediments. The relative sensitivity of the two procedures (guinea pig inoculation and microscopic observation) was thoroughly investigated. Thus, as early as 1932, Carvalho (*1*) demonstrated that the tubercle bacilli can be consistently detected by microscopy only when the concentration of bacilli in the sputum is about 100,000 bacilli per milliliter; in contrast, guinea pigs may develop progressive tuberculosis from the inoculation of a single bacillus (*2*). Guinea pig inoculation was therefore the most sensitive procedure in the diagnosis of tuberculosis and, naturally, became the criterion whereby one could determine the presence of active tuberculosis.

This picture was radically changed with the introduction of streptomycin and isoniazid in the treatment of tuberculosis in 1944 and 1952, respectively. Treatment with either drug resulted in a dramatic improvement in the clinical symptoms and in the rapid disappearance of the bacilli from the pathological secretions. However, relapses would occur because drug-resistant tubercle bacilli emerged. It was realized that the proper treatment of tuberculosis by chemotherapy required the laboratory demonstration of drug-sensitivity. Methods for culturing the tubercle bacilli developed earlier were adapted and widely used. This brought about two important consequences: one was the shift from the emphasis on guinea-pig inoculation; the other was the relatively frequent isolation of other mycobacteria whose pathogenic potential had to be ascertained.

In the diagnosis of tuberculosis it became necessary to determine the relative sensitivity of guinea-pig inoculation and the various culture procedures available. Comparative studies indicated that the culture methods were as sensitive as, if not more sensitive than, guinea-pig inoculation in the diagnosis of tuberculosis. Efforts were made to increase the sensitivity of culture methods by reducing the toxicity of digestion and decontamination procedures; this was also done by increasing the quality of available media or developing new media and by determining the optimal growth conditions. As a result of these efforts, guinea-pig inoculation became obsolete for diagnostic purposes.

In parallel with investigations on the bacteriological diagnosis of tuberculosis and monitoring of therapy by *in vitro* methods, a number of investigators addressed themselves to the question of the medical importance of other mycobacteria isolated from clinical materials. Although human disease caused by mycobacteria other than the tubercle bacilli was known before the era of chemotherapy, it was only recently that its medical and public health importance became recognized. Numerous cases began to be reported and the isolated bacteria were described. These mycobacteria were collectively named "atypical mycobacteria," a designation intended to mean a group of mycobacteria distinct from the typical tubercle bacilli. Landmarks in these investigations were reports in 1954 by Timpe and Runyon (*4*) and by Runyon (1959) who showed that the atypical mycobacteria could be arranged in four distinct groups on the basis of their growth characteristics. Runyon and his coworkers paved the road for detailed investigations in at least three areas of concern: the study of the taxonomic relationships of the mycobacteria, the definition of nosological entities, and the establishment of the epidemiological and public health importance of the mycobacterioses.

With the introduction of numerical methods of analysis in mycobacterial taxonomy, the field of mycobacteriology expanded rapidly. Species were defined and type or neotype strains became established, making it possible to deal with these bacteria on a sound scientific basis. Investigators working in cooperative international studies made great strides. These investigators addressed themselves not only to the question of definition of species but also to the foremost question of standardization of laboratory procedures of great relevance as a basis for international agreement in many aspects of public health.

Apart from the investigations centered in the medical and public health aspects of mycobacteriology, others concerned themselves with understanding the fundamental aspects of the biology of the mycobacteria, such as the energy requirements, intermediary metabolism, biosynthetic activities, structure and function, and genetics. Their observations contributed enormously to our understanding of the mycobacteria. Certainly, this understanding is necessary to the public health mycobacteriologist whose goal is the control and eventual eradication of the mycobacterioses. Someone called the tubercle bacillus the "Colossus of Bacteriology," and indeed we should treat this organism as such.

This book is an attempt to update earlier ones, and it covers what the author considers the major contributions of the last 20 years or so. The decision was made not to repeat information that can be found in previous books on the same subject. To establish the necessary continuity, however, some background information was found necessary on occasion.

Since this book deals with the biology of the mycobacteria exclusively, reference to other bacterial systems was kept to a minimum. There is what the author believes to be a good reason to do so. Indeed, the temptation to complete gaps in knowledge by resorting to analogy might have made the text comparable to texts in general bacteriology but would certainly make it non-provocative and misleading to the reader interested in finding out what is actually known about the mycobacteria. On the other hand, an understanding of the mycobacteria requires a straightforward description of how these organisms are handled in laboratory practice, which was also attempted. The author hopes to have succeeded in combining these two facets which would make the book a text for medical and public health workers, as well as a critical review of mycobacteriology useful for those engaged in research and development.

REFERENCES

1. Carvalho, A. (1932) Zeitsch. Tuberk. 63:305.
2. Doerr, R. and Gold, E. (1932) Zeit. Imm. Exp. Therapic. 74:7.
3. Runyon, E. (1959) Medical Clinics of North America 43:273.
4. Timpe, A. and Runyon, E. (1954) J. Lab. Clin. Med. 44:202.

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part 1

General Mycobacteriology

CHAPTER 1

Identification and Typing of the Mycobacteria

Introduction

The mycobacteria belong to the family of the *Mycobacteriaceae,* order *Actinomycetales* and class *Schizomycetes* (*12*). In the family *Mycobacteriaceae* two genera were recognized: *Mycobacterium* and *Mycococcus.* Recently, the genus *Mycococcus* was deleted from the family of the Mycobacteriaceae (*12a*).

The genus *Mycobacterium,* composed of rod-shaped cells that rarely branch under ordinary cultural conditions, is characterized by the unique staining property of acid-fastness, which is exhibited by individual cells. Although the property of acid-fastness is persistent, the ratio of acid-fast to non-acid-fast cells in a population may vary. The cells are prokaryotic and are surrounded by a rigid cell wall. They do not form flagella and other appendages (fimbriae, pili), capsules, or spores. Physiologically, the members of the genus are aerobic or microaerophilic. The type species is *Mycobacterium tuberculosis,* Lehman and Neuman (holotype: *M. tuberculosis,* strain H37Rv, deposited at the American Type Culture Collection, No. ATCC 27294). Recognized species are listed in Table 1.

Species are identified on the basis of cultural, physiological, and biochemical properties. Additional properties that are useful in identification are antigenic structure, sensitivity to toxic chemicals, susceptibility to bacteriophages, and pathogenicity for animal species.

Since numerical methods of analysis (*17,29*) were introduced in mycobacteriology, the field has rapidly expanded. Numerical taxonomic analysis permitted the selection of the laboratory tests most useful in discriminating between species. The first part of this chapter is concerned with the criteria and procedures evolved from these investigations that are useful in the identification of species of mycobacteria commonly isolated from clinical materials. The second part deals with subdivisions within the species, or typing. In the last part of the chapter, the biochemical tests useful in the clinical laboratory are described in detail. Methods recommended for isolation from clinical specimens are described in Part 2.

TABLE 1. Alphabetical Listing of Accepted Names of Mycobacterial Species

Accepted Names	Type or Neotype Strain, Some Usual Synonyms
M. avium Chester, 1901 (12)	*M. tuberculosis* var. *avium*; avian tubercle bacilli, ATCC 25291
M. bovis Karlson and Lessel, 19,70 (79)	*M. tuberculosis* var. *bovis*; bovine tubercle bacilli, ATCC 10210
M. chelonei Bergey *et al.* 1923 (12)	*M. absessus,* NCTC 947, *M. borstelense* (23), *M. runyonii*
M. diernhoferi Bönike and Juhasz, 1965 (22)	ATCC 19340
M. flavescens Bojalil, Cerbón and Trujillo, 1962 (17)	ATCC 14474
M. fortuitum da Costa Cruz, 1938 (12)	*M. ranae,* ATCC 6841, *M. giae, M. minetti, M. peregrinum*
M. gastri Wayne, 1966 (184)	ATCC 15754
M. gordonae Bojalil, Cerbón and Trujillo, 1962 (17)	*M. aquae*; tap water scotochromogens, ATCC 14470
M. intracellulare (Cuttino and McCabe) Runyon, 1967 (145)	Battey bacilli, ATCC 13950 (Yandle), *M. brunense*
M. kansasii Hauduroy, 1955 (66)	*M. luciflavum*; yellow bacilli, ATCC 12478 (Bostrom)
M. leprae (Hansen) Lehmann and Neumann, 1896 (12)	Hansen's bacilli
M. lepraemurium Marchoux and Sorel, 1912 (12)	
M. marinum Aronson, 1926 (12)	*M. balnei, M. platypoecilus,* ATCC 927 (Aronson)
M. microti Reed, 1957 (12)	*M. tuberculosis* var. *muris*
M. nonchromogenicum Tsukamura, 1965 (174)	ATCC 19530, *M. terrae* (Tsukamura).
M. paratuberculosis Bargey *et al.*, 1923 (12)	*M. johnei*
M. phlei Lehmann and Neumann, 1899 (12)	ATCC 11758 (suggested working type) *M. moelleri*
M. scrofulaceum Prissick and Masson, 1956 (133)	ATCC 19981, *M. marinum*
M. smegmatis (Trevisan) Lehmann and Neumann 1899 (12)	*M. butyricum,* ATCC 14468
M. terrae Wayne, 1966 (184)	Group III radish, ATCC 15755
M. thamnopheos Aronson, 1929 (12)	ATCC 4445
M. triviale Kubica *et al..,* 1970 (94)	ATCC 23292
M. tuberculosis (Zpof) Lehmann and Neumann, 1899 (12)	*M. tuberculosis* var. *hominis,* human tubercle bacilli, Koch's bacilli, ATCC 27294 ($H_{37}Rv$)
M. ulcerans MacCallum, 1948 (106)	*M. buruli*
M. vaccae Bönicke and Juhasz, 1964 (22)	ATCC 15483
M. xenopi Schwabacher, 1959 (150)	*M. littorale,* ATCC 19250

Identification of the Mycobacteria

BASIC PRINCIPLES IN IDENTIFICATION

The criteria and procedures described are those adopted at the Center for Disease Control (CDC). The reason for this selection is not that other criteria or procedures are necessarily less reliable, but that the diagnostic scheme described has proved satisfactory for medical and public health purposes.

Once isolated in pure cultures, the mycobacteria can be grouped according to the time and temperature of incubation required to obtain visible growth and according to the appearance of the colonies (colony type and whether pigment is present). Initially, these observations can be made on primary isolation, provided that the cultures are observed at weekly intervals. However, the purity of the culture should be assessed first. Purity is established by streaking a sample of the culture onto the surface of agar base media to obtain well-isolated colonies, and purity means both: 1) the absence of contaminating organisms; and 2) the absence of a mixed culture of mycobacteria.

Although *M. tuberculosis* is the species of primary concern in medical and public health mycobacteriology and its presence in a culture can be inferred from the incubation time required for visible growth[1], the appearance of the colonies, and the demonstration of niacin in culture extracts, the bacteriologist must study the organisms to arrive at a final diagnosis.

Isolated colonies of each type must be transferred, preferably into a liquid medium and studied separately. Cultures thus obtained are then transferred to a variety of media to determine their rate of growth, pigment production, and other properties necessary for identification.

On the basis of their rate of growth and pigment formation, Runyon (*144*) divided the mycobacteria usually isolated in the clinical laboratory (except *M. tuberculosis, M. bovis, M. avium,* and *M. marinum*) into four groups. These groups are described in Table 2. Most of these mycobacteria, hitherto referred to as "atypical" or "unclassified," can now be speciated, and only those that current methods fail to speciate should be grouped according to Runyon. The frequency of unidentifiable strains received in our laboratory was, from 1969 throughout 1971, 0.8% for Group II, 1.1% for Group III, and 4.9% for Group IV.

TABLE 2. Grouping of Unclassified, Anonymous or Atypical Mycobacteria [according to Runyon, *141*]

Group	Description
I Photochromogens	Slow grower. Colony mass nonpigmented if grown in dark; bright yellow within 6 to 24 hours when exposed to visible light when young.
II Scotochromogens	Slow grower. Colony mass yellow to orange, even if grown in dark.
III Nonphotochromogens	Slow grower. Pigment in cell mass usually absent; if present, it accumulates slowly with age.
IV Rapid growers	Growth in 7 days or less.

---

[1] Sometimes called growth rate. This expression should not be confused with the same expression as used in general bacteriology (see pages 10, 54). To avoid confusion, the designation "rate of growth" is used instead.

The properties of the mycobacteria most commonly isolated in the clinical laboratory are listed in Table 3. The fundamental aspects of each of the indicated tests are discussed below. These tests meet the criteria Wayne (*181*) set forth as necessary to their widespread usage in diagnostic laboratories. According to the criteria, the test must: 1) require a minimum of handling of large masses of bacilli; (2) employ a simple technical procedure; 3) provide rapid results; and 4) allow a minimum of ambiguity in interpretation.

MORPHOLOGY AND STAINING PROPERTIES

The mycobacterial cells are rod shaped, ranging in length from 0.5 μ to 4 μ or more and in width from 0.3 to 0.6 μ. Young cultures may exhibit rudimentary branching that is conspicuous in *M. intracellulare* grown in HeLa cells (Fig. 1). Cells of *M. tuberculosis* tend to remain parallel to each other, forming tightly packed cords, a pattern that is associated with the presence of a toxic glycolipid ("cord factor") and with virulence (see pages 45,123-124).

The mycobacterial cells are difficult to stain, but, once stained, strongly retain the colorants which are not removed by treatment with dilute solutions of strong mineral acids. This property, known as acid-fastness, is demonstrated by the Ziehl-Neelsen staining procedure, of which there are various modifications. Acid-fastness is related to the nature of the complex lipids found in these cells, in particular the branched hydroxy-acids of high molecular weight, which are known as mycolic acids, and to the physical integrity of the cells. The mycolic acids are acid-fast (*4*), but they are not the only factor in the characteristic property, because alterations of the cells' permeability (*199*) or mechanical disruption by grinding (*155*) and sonication (*98*) that do not alter the mycolic acids do cause a loss in acid-fastness. Irradiation of fixed smears with ultraviolet light also causes loss in acid-fastness (*117,118,119,120*), and the rate of loss differs among species.

Once stained with fluorescent dyes, the mycobacteria fluoresce strongly. After an initial report by Hagemann, acid-fast staining procedures with fluorescent dyes were developed (*14,15*). Truant's modification (*173*) of Hagemann's procedure has been successful. The lack of background in most procedures has been obviated in a recent modification in which acridine-orange is used as a true counterstain (*160*).

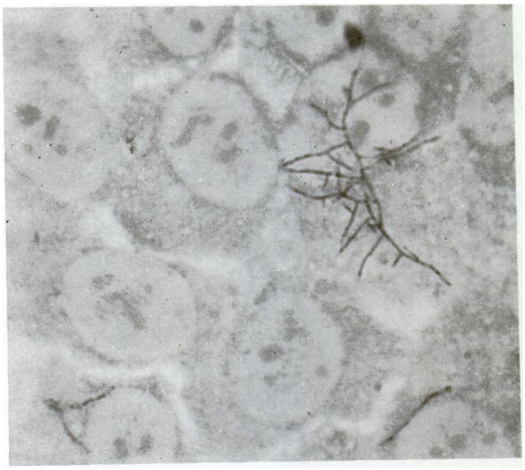


FIGURE 1. Branching of *M. intracellulare* grown in HeLa cells (adapted from Brosbe, Sugihara, and Smith, *27*).

TABLE 3. Characteristics of Mycobacteria Commonly Isolated from Clinical Materials

[Note that other mycobacteria which fall into Runyon's Groups II, III, and IV, are also isolated but cannot be identified as to the species by current methods. Temperature relationships are indicated in Table 4.]

Species	Runyon's group ("atypical", "anonymous") mycobacteria	Rate of growth (days)	Pigment formation		Niacin	Nitrate reduction	Catalase			Tween hydrolysis (days)		Tellurite reduction	Arylsulfatase		Growth on				Colony type on 7H-10 agar
			Dark	1 hr. light			<40 mm	>50 mm	pH 7/68°C	5	10		3 days	2 weeks	5% NaCl	Mac-Conkey agar	TCH	Tb_1	
M. tuberculosis	N.A.	Slow (12-30)	–	–	+	3+	+	–	–	∓	∓	–	–	–	–	–	+	–	Rough-R
M. bovis	N.A.	Slow (25-40)	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	Rough-R
M. avium [1]	N.A.	Slow (10-21)	–	–	–	–	+	–	+	–	–	+	–	–/1+	–	–	+	+	Smooth-I or Rough-R
M. marinum	I	Intermediate (7-11)	–	+	∓	–	±	∓	–	+	+	–	–/2+	4+	–	–	+	+	Smooth-S or K-like
M. kansasii	(Photochromogens)	Slow (10-21)	–	+	–	3+	+	+	+	+	+	–	–	–/1+	–	–	+	–	Smooth-K or Rough-R
M. scrofulaceum	II	Slow (10-30)	+	+	–	∓	+	+	+	–	–	–	–	–	–	–	+	+	Smooth-I or Rough-R
M. gordonae	(Scotochromogens)	Slow (10-25)	+	+	–	–	+	+	+	+	+	–	–	±	–	–	+	+	Smooth-I or Rough-R
M. flavescens		Intermediate (10-14)	+	+	–	3+	+	+	+	+	+	–	–	–/4+	±	–	+	+	Smooth-S or Rough-R
M. intracellulare [1]		Slow (10-21)	–	–	–	–	+	–	+	–	–	+	–	–/2+	–	∓	+	+	Smooth-I or Rough-R
M. gastri	III	Slow (10-21)	–	–	–	–	+	–	–	+	+	–	–	–	–	–	+	+	K-like
M. terrae		Slow (10-21)	–	–	–	3+	+	+	+	+	+	–	–	–/2+	–	–	+	+	Smooth-I or Rough-R
M. triviale	(Non-chromogens)	Slow (10-21)	–	–	–	3+	+	+	+	+	+	–	∓	3/5+	+	–	+	+	Rough-R
M. xenopi		Slow (10-35)	+	+	–	–	+	–	+	–	–	–	+	2/5+	–	–	+	+	Smooth-X or Rough-X
M. ulcerans [2]	N.A.	Slow (90-120)	–	–	±														
M. africanum [3]	N.A.	Slow (12-30)	–	–	±	–/1+	+	–	–	–	–	–	–	–	–	–	–	+	Rough-R
M. fortuitum	IV	Rapid (3-5)	–	–	–	±	+	+	+	–	∓	∓	±/2+	3/5+	+	+	+	+	Rough-R or Smooth-S
M. smegmatis	(Rapid growers)	Rapid (3-5)	–	–	–	+	+	+	+	+	+	+	–	–	–	–	+	+	

---

[1] The scheme does not distinguish *M. avium* and *M. intracellulare* (see text).

[2] Not investigated, mainly because of the difficulty in obtaining enough bacterial mass in vitro.

[3] ***M. africanum*** has not yet been recognized as a separate species. Its properties are intermediate to ***M. tuberculosis*** and ***M. bovis.***

– Properties enabling the inclusion of a strain in the indicated grouping.

– Key tests for discriminating within each of the indicated groupings.

Fluorescent antibody methods were described (*33,78,112,124*), but they were not extensively used because of the difficulty in obtaining suitable sera in enough quantities for general distribution.

Recommended staining procedures are described in Part 2, pages 149-154.

TEMPERATURE RELATIONSHIPS

On the basis of the temperature at which the bacteria grow best, they can be classified as thermophiles (better growth at 40°C or more), mesophiles (better growth in the range of 20°C to 37°C), and psychrophiles (better growth at 20°C or less). All mycobacteria, at least those isolated from man and other animals, are mesophiles. Yet, the best temperature of incubation on primary isolation may be high (*M. avium* and *M. xenopi*) or relatively low (*M. marinum* and *M. ulcerans*). With the possible exception of *M. ulcerans,* all other mycobacteria grow well at 35°-36°C on subculture in the laboratory. Temperature relationships have been established for several of the mycobacteria, generally by observing the amount of growth of cultures incubated at various temperatures (Table 4). More precise information is obtained by determining the effect of temperature upon the growth rate (Fig. 2), a procedure that determines the optimal temperature of growth defined as the temperature at which the growth rate is maximal. Unfortunately, no detailed studies have been conducted on the temperature relationships throughout the genus.

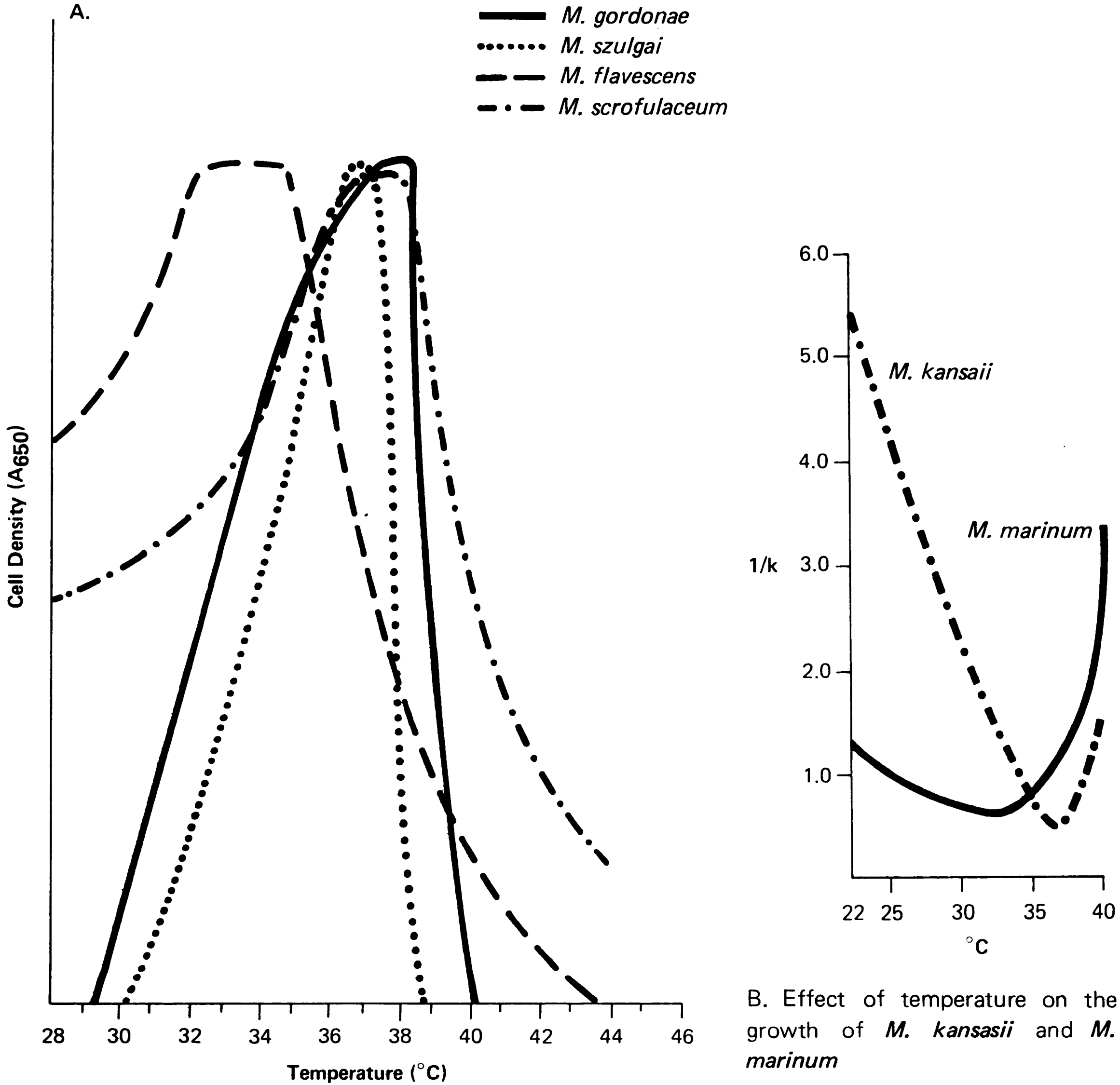
TABLE 4. Temperature Relationships of Some Mycobacteria

Species	**Temperature of Incubation (°C)**						**References**
	20-22	**30-33**	**37**	**40**	**44**	**45**	
M. tuberculosis	–	±	+	–	–	–	36
M. bovis	–	±	+	–	–	–	36
M. avium	–	+	+	+	+	±	13,36,49,65
M. marinum	±	+	+	–	–	–	35,156
M. kansasii	±	+	+	±	–	–	16,36,65,128,195
M. scrofulaceum	±	+	+	±	–	–	16,36
M. gordonae	±	+	+	±	–	–	36
M. intracellulare	±	+	+	+	±	–	16,36,128
M. xenopi	–		+	+	+	+	16,36,53,59,128
M. fortuitum	+	+	+	+	–	–	7,36,63
M. phlei	+	+	+	+	+	+	7,36,63
M. smegmatis	+	+	+	+	+	+	7,36,63
M. ulcerans	–	+	±	–	–	–	34,106

PIGMENT PRODUCTION

The mycobacteria synthesize carotenoid pigments that are very conspicuous in some species. In some, pigment production is synthesized only after young cultures are exposed to visible light, and these were named photochromogenic mycobacteria; in others, coined scotochromogens, the pigment is synthesized whether the cultures are incubated in the dark or in the light; finally, a third group does not synthesize pigment in conspicuous amounts, and these are called nonchromogenic mycobacteria. Runyon proposed the above designations, which characterize his Group I (photochromogenic mycobacteria), Group II (scotochromogenic mycobacteria), and Group III (non-chromogenic mycobacteria) (Table 2).

In studying the conditions of pigment production in the diagnostic laboratory, duplicate cultures are prepared, and one is incubated, shielded from visible light. When visible colonies appear on the unshielded culture, the shield is removed from the other culture, and the presence of pigment is recorded. If no pigment is observed, the colonies are exposed to light for 1 hour, and the culture is then incubated shielded overnight. The production of pigment after light exposure indicates that the culture is a photochromogenic



A. Growth of the mycobacteria and the effect of temperature (original).

B. Effect of temperature on the growth of *M. kansasii* and *M. marinum*

The reciprocal of the growth rate constant, K, is plotted against the temperature of incubation in centigrades, °C.

FIGURE 2.

Mycobacterium species. In determining pigment production, overinoculation may give erroneous results. In addition, pigment production in both the scotochromogens and the photochromogens requires free access of air and, further, only young cultures of the photochromogens produce pigment (*189*).

In general, the scotochromogenic mycobacteria when grown in the dark, produce a bright yellow pigment that may deepen to orange and, rarely to brick red if exposed to light continuously for 2 weeks; the photochromogenic mycobacteria produce a lemon-yellow pigment only if exposed to light, and bright orange crystals may often be observed in *M. kansasii* colonies exposed continuously to light. The mechanism of β-carotene synthesis in *M. kansasii* and *M. marinum* is discussed in Part 1, Chapter 3, pages 62-63, 66-67.

RATE OF GROWTH

Rate of growth is currently determined by plating the surface of a suitable solid medium with an inoculum light enough to yield isolated colonies. The cultures are incubated and read 5 to 7 days after plating, and thereafter at weekly intervals. Rapid growing mycobacteria yield mature colonies within 7 days or less of incubation; those that yield colonies in 7 days or more are referred to as slow growers.

COLONY MORPHOLOGY

The mycobacteria form a variety of colony types on solid media. The colonial morphology may change with the medium, but in any given one, it is remarkably stable. The systematic study of colony types in mycobacteriology began with a report by Fregnan, Smith, and Randall (*55*), who used the Dubos oleic acid-albumin-agar medium because of its transparency. These investigators sought to obtain homogeneous populations for the isolation of certain species-specific glycolipids, the mycosides (page 45). They demonstrated a close relationship between the colony morphology and the mycoside content of the cells. Soon afterwards, Fregnan and Smith (*56*) proposed a systematic classification of colony types and emphasized the importance of the medium chosen. They used the words "smooth" and "rough," followed by symbols, to describe the colony types. For example, "rough-Cs." indicates a rough colony with a corded structure; "smooth-S" denotes a smooth colony with a central cone which gradually becomes flat toward the periphery. Following these initial reports, Kubica and his coworkers *(77,92,96,178)* described the colony morphology that the mycobacteria, commonly isolated in clinical laboratories, yield on oleic acid-albumin-agar, corn meal agar, and Middlebrook's 7H10 agar. The classification shown in Table 5 is adopted from Vestal and Kubica (*178*), and it applies to the colony forms on the 7H10 agar medium. The terminology follows that of Fregnan and Smith, although the symbols are not necessarily the same as theirs. Illustrative pictures are shown in Figure 3.

On all media, *M. tuberculosis* forms dry, rough, friable colonies which are distinct from the rough colonies formed by other species, yet not so distinct as to be pathognomonic. Lorian (*104*), showed that the cording observed in young colonies of *M. tuberculosis* but not of others, could be enhanced by adding the tensioactive agent Triton WR-1339 to the 7H10 medium. Mature colonies of *M. xenopi* are very distinct; some of these colonies may be partially or completely covered by short aerial hyphae-like structures. Colonies with aerial hyphae are rough; smooth colonies rarely, if ever, exhibit aerial hyphae and are moist and

TABLE 5. Mature Colony Forms of Mycobacteria on 7H10 Agar
[adapted from Vestal and Kubica, *175*]

Colony Form	Species
Rough R or Ry	*M. tuberculosis* *M. bovis* *M. kansasii* *M. fortuitum* Group II Group III Group IV *M. triviale*
Smooth S or Sy	Group II Group III *M. avium*
Smooth D or Dy	Group II Group III *M. avium* Group IV
Smooth T	Group III *M. avium*
Smooth Kw	*M. kansasii*

translucent (*146*). *M. bovis* forms small, translucid, colorless, and pyramid-shaped colonies on egg medium. These few examples show how useful the recognition of colony types is in identification. In addition, the ability to recognize colony types is also important in determining contamination and mixed cultures. Regarding the latter, one must bear in mind that some mycobacteria almost consistently yield more than one colony type on a plate. An example is *M. intracellulare.*

BIOSYNTHESIS OF NICOTINIC ACID (NIACIN)

In an investigation on the biosynthesis of Complex B vitamins in *M. tuberculosis* and *M. bovis,* Pope and Smith (*132*) noticed that the amount of nicotinic acid accumulated in filtrates of cultures of the former was much higher than in filtrates of the latter. Later comparisons of the amount of total niacin produced by various mycobacteria indicated that *M. tuberculosis* produced the largest amounts (Table 6), and therefore, the accumulation of niacin could serve as a means of diagnosing this species (*84,85,86*). This finding was conclusively confirmed in later reports by other investigators. Following these early reports, the test for the identification of niacin became one of the most important and extensively used tests in mycobacteriology.

Niacin is a component of the coenzymes nicotinamide adenine dinucleotides (NAD and NADP) that play a vital role in the oxidation-reduction reactions in all living cells (Chapter 3). In man and other mammals, it is an essential dietary requirement. In organisms, including some bacteria, that depend on it as a vitamin or growth factor, its only physiological function is to serve as a precursor in the biosynthesis of the coenzymes. In the mycobacteria, NAD and NADP seem to be synthesized either from nicotinic acid (*42,43*) or from endogenous quinolinic acid (*3,52,87,142*) via the intermediate nicotinamide adenine dinucleotide (Fig. 4). In these bacteria tryptophan is not a precursor of niacin (*87,142*), and the pathway for its biosynthesis is similar to the one described in *Bacillus subtilis* (*198*).

The mechanism of excess production of niacin in *M. tuberculosis* has been viewed differently by Konno, and Dudley and Willet. According to Konno, the cell-free extracts of tubercle bacilli are incapable of converting the niacin to nicotinic acid mononucleotide (reaction E-2 in Fig. 4), in contrast to extracts of other mycobacteria (a photochromogenic strain, *M. avium,* and *M. smegmatis*). The excess niacin accumulation was therefore due to a blocked pathway, which resulted in the inability to reutilize endogenous nicotinic acid found in reactions 5 or 6 when ATP is limiting. On the other hand, Dudley and Willet observed that NAD was synthesized from niacin by cell-free extracts of both *M. tuberculosis* and *M. bovis.* According to these workers, the excess production of niacin in *M. tuberculosis*

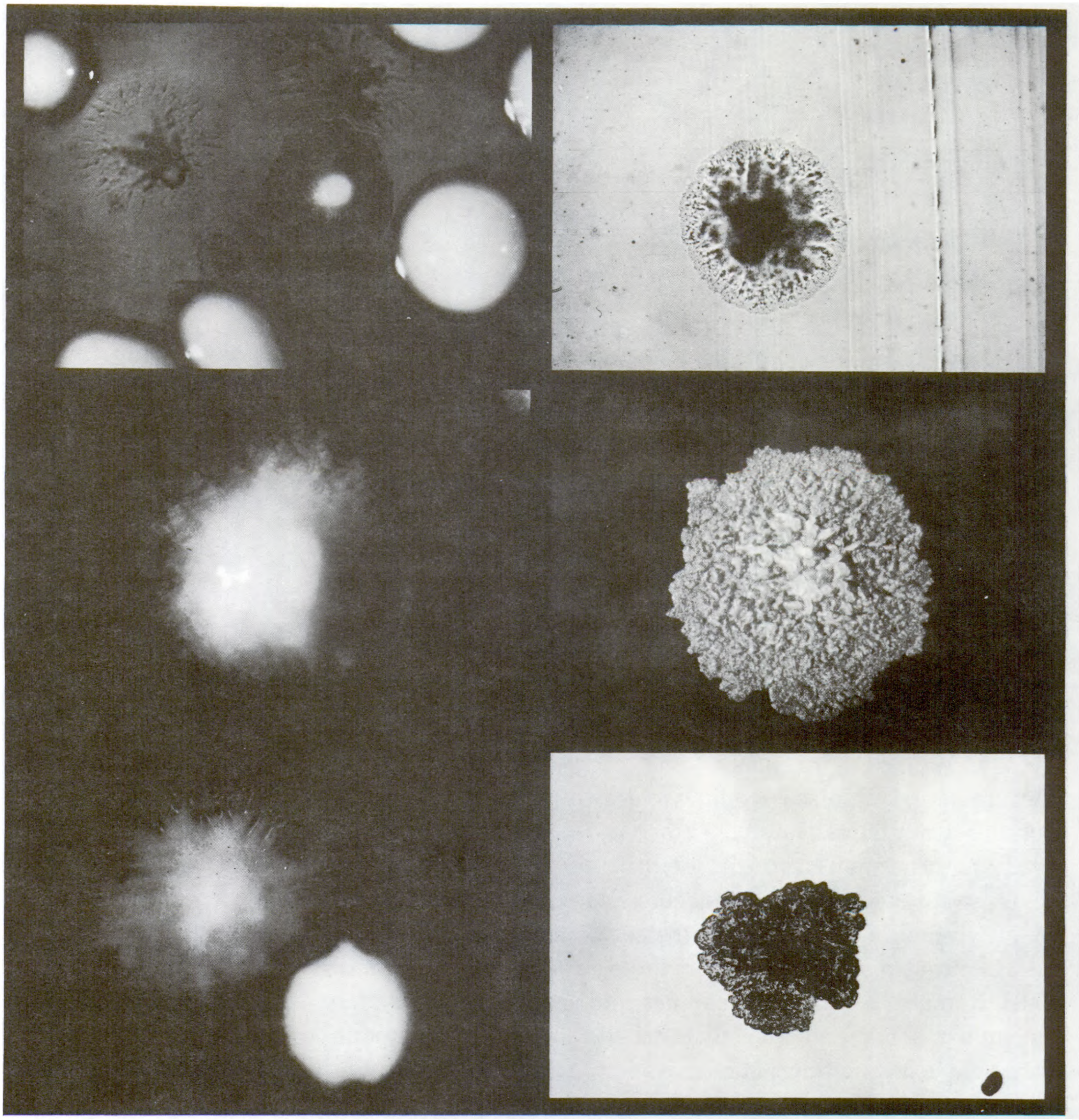


FIGURE 3. Mycobacterial colony types.

TABLE 6. Nicotinic Acid Production by Mycobacteria

Species	**Strain**	**Nicotinic acid[1] in culture filtrate (γ/ml)**	**Nicotinic acid[1] in dried cells (γ/mg)**
M. tuberculosis	$H_{37}Rv$	30.0	12.4
	Campbell	13.5	6.7
	Erdman	22.0	10.2
	Pearson I	16.0	6.9
	$H_{37}Ra$	26.0	11.3
	JH16Ra	32.0	8.4
	JH6a	15.0	6.3
M. bovis	Vallee	1.0	0.3
	Ravenel	1.5	0.5
	BCG	1.0	0.4
M. avium	Sheard	1.0	0.4
	Camden	2.0	0.9
	Avian I	1.0	0.6
M. fortuitum	*M. ranae* from Trudeau Laboratory	1.0	0.3
M. phlei	From Trudeau Laboratory	1.8	0.7
M. smegmatis	ATCC 607	2.0	0.8

[1] Before assaying for niacin, the culture filtrates and the dried cells were hydrolyzed with sodium hydroxide. The values in the table represent, therefore, total niacin (free niacin and niacin present in coenzymes, nicotinamide, and putative precursors).

is due to the degradation of accumulated nicotinic acid mononucleotide which is greater than in *M. bovis* because of less efficient utilization in the synthesis of NAD. Their data suggest that reaction E-3 (Fig. 4) is the rate-limiting step in *M. tuberculosis.*

In the usual manner, niacin is identified by reacting it with cyanogen bromide in the presence of an aromatic amine. The product of the reaction of niacin with cyanogen bromide reacts in turn with the aromatic amine to give a colored product. The mechanism of the reaction and the color of the product with some aromatic amines is shown in Figure 5. Many other aromatic amines tested varied considerably in their sensitivity (*201*). To simplify the procedure, Kilburn and Kubica (*80*) developed a reagent-impregnated paper strip whose reliability has since been confirmed (*39,58*) and which is currently used in routine diagnosis. Technical details in performing the tests are described on page 26-28.

NITRATE REDUCTION

Some mycobacteria can utilize nitrate and nitrite as sources of nitrogen, replacing ammonium salts (*44,67,177*). Virtanen (*180*) investigated the ability of intact cells to reduce nitrate to nitrite and observed that the mycobacteria differed quantitatively in nitrate reduction to such an extent that the reaction could be useful for identification. In his very extensive monograph, Virtanen analyzed the various factors influencing the reaction, such as, the age of the culture, hydrogen ion concentration, temperature, enzyme inhibitors, etc.

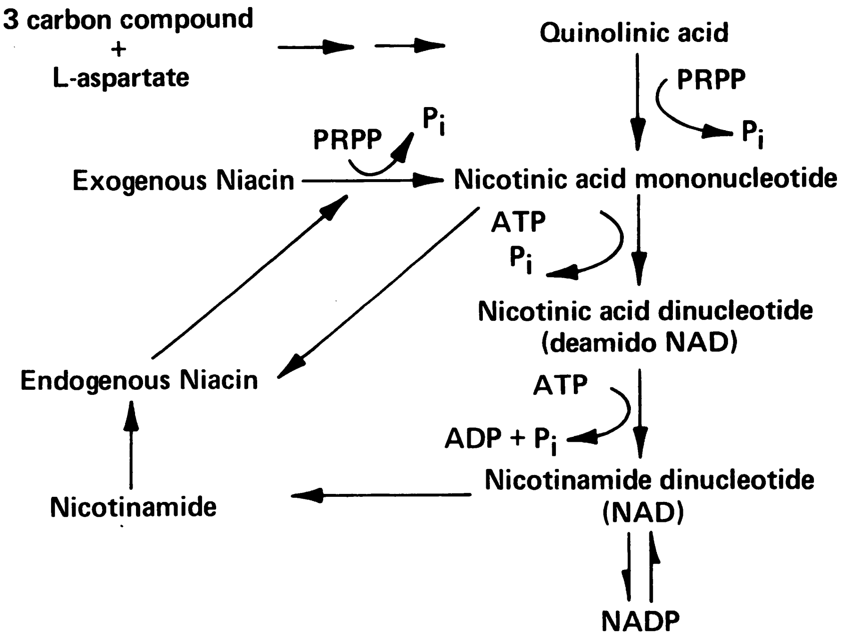
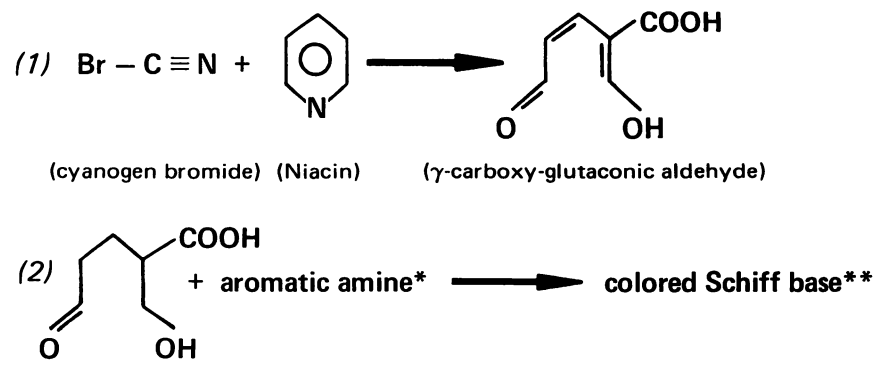


FIGURE 4. Hypothetic biosynthetic pathway in the synthesis of NAD and NADP in the mycobacteria.



Procedures:

A. Test tube

1. Cyanogen bromide + niacin → γ-carboxy-glutaconic acid aldehyde
2. γ-carboxy-glutaconic acid aldehyde + aniline → Yellow color

B. 1. Chloramine-T + Potassium Thiocyanate → Cyanogen chloride

2. γ-carboxy-glutaconic aldehyde + p-aminosalycilate → yellow color

(*) (**)

Aromatic amine	*Color found*
Aniline	yellow
Benzidine	violet-pink
o-toluidine	coral red
p-amino salycilate	yellow
p-aminobenzoate	yellow

FIGURE 5. Proposed mechanism of the reaction currently used in the identification of niacin (*51*).

The nitrate reduction test is particularly useful in discriminating *M. tuberculosis* and *M. bovis.* The former exhibits a strong activity, and the latter gives a negative or weakly positive reaction. However, both organisms synthesize the enzyme nitrate reductase. As Virtanen (*180*) noted, the differences in the abilities of intact cells of the mycobacteria to reduce nitrate are quantitative rather than qualitative. An analysis of this phenomenon follows.

DeTurk and Bernheim (*38*) reported that *M. bovis* (BCG) assimilated nitrate and nitrite after a latent period, and that the synthesis of the enzymes was adaptive. Thus, the latent period was shortened by preincubating the cells with nitrate or nitrite, and the synthesis of nitrite reductase was inhibited by aureomycin and streptomycin when these were added with the substrate, but they were without effect when added at the end of the latent period. The adaptive assimilation of nitrite was inhibited by ammonium ion until this ion was completely utilized (Fig. 6). These observations indicated that the latent period of assimilation corresponded to the time the protein enzymes were induced (inducible enzyme synthesis).

Intact cells of *M. tuberculosis* (H37Ra) reduce nitrate without a lag period (Fig. 6), the enzyme being constitutive. Nitrate reductase is present in a particulate fraction obtained by differential centrifugation of mechanically disrupted cells. Reduction of nitrate by the particulate enzyme requires the addition of NADH, while NADPH, succinate, lactate, formate, and pyruvate do not serve as electron donors. Thus, the nitrate reductase was found to be NADH-dependent (*8*).

In the mycobacteria, nitrate does not serve as a final electron acceptor and cannot substitute for molecular oxygen. In relation to energy yielding oxidation reactions, it serves as a nonessential electron acceptor, thus interfering with the normal energy yielding reactions of the cells. However, as noted, it may be assimilated, the nitrogen being used for protein synthesis.

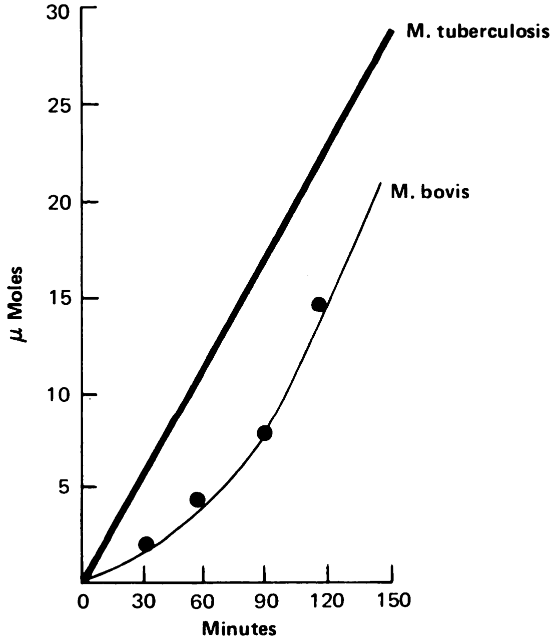


FIGURE 6. Nitrate reductase of *M. tuberculosis,* strain $H_{37}Ra$, and *M. bovis,* BCG strain ATCC 8420. Data from, respectively, Bastarrachea and Goldman (*8*) and DeTurk and Bernheim (*38*).

The NADH-dependency of nitrate reductase may explain more recent observations by Bönicke and Kazda (*24*) and Bönicke, Juhasz, and Diemer (*26*) who reported that the reduction of nitrate or nitrite was increased by adding oxidizable substrates to the reaction mixture.

In regard to their accessibility to the substrate, enzymes in intact cells may be cryptic because of permeability barriers to the passage of the substrate. The available information suggests that the quantitative differences among intact cells of the mycobacteria in their ability to reduce nitrate are related to their permeability to the substrate rather than to lack of genetic information for synthesizing the protein enzymes. This and other related questions remain unanswered.

CATALASE ACTIVITY

All mycobacteria synthesize catalases. The usual way to demonstrate catalase activity is to add one drop of an equal mixture (v/v) of 10% Tween 80 and 30% hydrogen peroxide to a culture slant (*114*). Bubbles are produced almost immediately after the substrate is added. Catalase is an intra-cellular soluble enzyme, and there is more than one kind of catalase protein in respect to heat stability. Quantitative differences in catalase activity by intact cells and differences in heat stability (see Part 1, Chapter 3) were used to advantage in developing tests useful in the diagnostic laboratory (*89,181*).

In Wayne's semiquantitative test (*188*), the height of the column of bubbles produced is less than 45 mm in *M. tuberculosis, M. bovis, M. avium-intracellulare, M. xenopi,* and *M. gastri.* According to Wayne, strains of *M. kansasii* that produce more than 50 mm of bubbling are usually clinically significant. The enzyme protein is heat labile in Kubica and Pool's test (*89*) for *M. tuberculosis, M. bovis, M. gastri* and most strains of *M. marinum.*

Isoniazid-resistant mutants of *M. tuberculosis* are generally catalase negative (*114*). Besides loss of catalase activity, other changes in the tubercle bacilli are associated with the acquisition of resistance to isoniazid. This subject is discussed in Part 2, Chapter 1, pages 123-124.

ARYLSULFATASE

The arylsulfatases are enzymes that hydrolyze compounds with the general formula $R \bullet OSO_3H$, in which R is an aromatic structure. The detection of arylsulfatase activity in various mycobacteria and other bacteria was reported by Whitehead and his coworkers (*193,194,200*). These investigators used either potassium phenolphthalein disulfate or napththylsulfate as substrates. Phenolphthalein is the product of the reaction with the former and is detected by the addition of alkali; the product of the reaction with the latter substrate is naphthol, which is detected by a diazo reaction. Because diazo reactions were observed in culture media even in the absence of the substrate, potassium phenolphthalein disulfate was considered the most convenient of the two.

The hydrolysis reaction is usually studied by growing the cells in a medium containing the substrate. After a suitable period of incubation, free phenolphthalein is detected by the red color that forms when an alkali is added. The amount of free phenolphthalein detected depends upon the substrate concentration and the time of incubation (*90*). The time of incubation may simply represent the time it takes for the population to attain a critical cell mass, or it may be a composite of the former with a latent period of enzyme induction. As Tarshis (168) showed, the cell mass is certainly an important factor. A negative test may be due to lack of the enzyme or to its cripticity; in some mycobacteria, for example *M.*

marinum, the enzyme appears to be adaptive. However, the conditions for the synthesis of the enzyme have not been investigated.

Kubica and his coworkers (*90,91,92*) did systematic studies to establish the usefulness of the test, to define the testing conditions, and to evaluate its reproducibility. The procedure described on pages 32-33 is, to a large extent, the result of their investigations.

HYDROLYSIS OF TWEEN 80

Davis and Dubos (*37*) reported that the mycobacteria contain enzymes capable of hydrolytically releasing oleic acid from the poly-oxethylene derivative of sorbitan mono-oleate Tween 80. Further studies (*6*) with cell-free extracts demonstrated that mycobacteria may contain distinct esterases as judged from their specificity towards various substrates. Although the use of distinct substrates is of potential value in identification, only Tween 80 has been used for this purpose. The testing procedure in which Tween 80 is used as the substrate (developed and evaluated by Wayne (*182,189*) is particularly useful in discriminating *M. scrofulaceum* (negative) from *M. gordonae* and *M. flavescens* (positive), and in discriminating among the nonchromogens.

TELLURITE REDUCTION

Gosio (*64*) reported that mycobacterial cells reduced tellurite salt to metallic tellurium. Cytochemical investigations in sections of bacteria treated with potassium tellurite revealed that the metal is selectively deposited at the membrane, probably on the mesosomes (*148*). The sites of deposition appear to relate to areas of oxidation-reduction activity, corresponding to the localization of the terminal respiratory chain in the membrane (*115,171*). Figure 7 is an electron microscopy picture of *M. avium* cells grown on a collodion film and then replaced on a medium containing potassium tellurite. Fine needles or small particles of metallic tellurium are clearly visible in localized areas. Tellurite serves as an artificial electron acceptor, thus being reduced to metallic tellurium. Tellurite reductase was found as a soluble enzyme in *M. avium* (*169*).

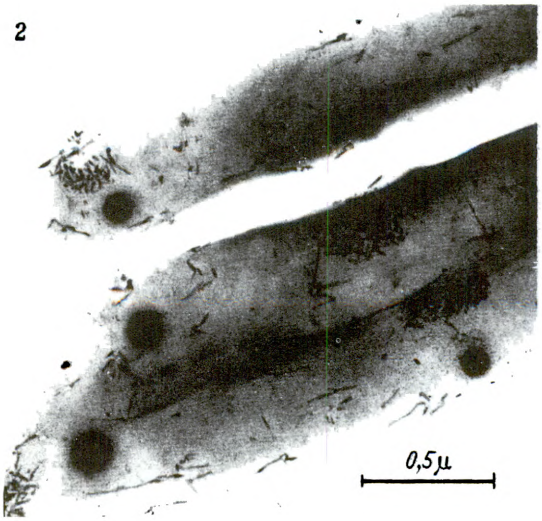


FIGURE 7. Fine needle-like crystals of reduced tellurite are deposited in reduction sites. The large, dense bodies are polyphosphate granules (adapted from Toda, Tokeya and Koike, *171*).

Kilburn, Silcox and Kubica (*82*) observed that adding a solution of potassium tellurite to cultures of mycobacteria in liquid medium close to the stationary phase of growth resulted in variable rates of reduction. They concluded that a 3-day test was of greatest value and highest reliability, especially in the study of nonchromogenic slow-growing mycobacteria (Runyon's Group III).

GROWTH ON MEDIA CONTAINING INHIBITORY AGENTS

The ability of mycobacteria to grow on media containing toxic substances is of differential value, and a number of substances have been used for this purpose. The following is a partial list of reported growth inhibitory agents: picric acid (*183*), neotetrazolium (*59*) 8-oxiquinoline (*165*), hydroxylamine (*176*), 8-azaguanine (*74*), various dyes (*73*) and p-nitro-2-acetylamino-β-hydroxy-propiopthenone (*72*).

Four of the procedures listed in Table 3 use this principle, namely, the ability to grow on MacConkey's agar, on medium containing thiacetazone (Tb_1), on medium containing thiophen-2-carboxylic acid hydrazide, and on medium containing 5% sodium chloride.

Gordon and her coworkers approached the problem of classifying rapid growing mycobacteria by using physiological and cultural characteristics (*61,62,63*). The ability of the organism to grow in a variety of media was found to be of some differential value. Jones and Kubica (*73*) observed that most *M. fortuitum* strains were capable of growth on MacConkey agar, although most *M. smegmatis* strains failed to grow. The frequency of strains of *M. smegmatis* that grow on the medium depends upon the size of the inoculum and the temperature and time of incubation, and the differential value of MacConkey agar is increased by standardizing the size of inoculum and the plating procedure.

The size of the inoculum is a critical factor in all tests that are based upon ability to grow in the presence of toxic substances, whether these can be used as chemotherapeutic agents or not. The chemical thiacetazone (conteban, Tb_1) is extensively used in the treatment of tuberculosis in many countries, but not in the United States. Wolinsky, Smith, and Steenken (*196*), and Bönicke (*19*) showed that the various mycobacteria differed in their susceptibility to thiacetazone and other thiosemicarbazones, and Vestal and Kubica (*179*) proposed the use of thiacetazone at a concentration of 10 micrograms per milliliter in an agar base medium as a means of differentiating the mycobacteria. In this context, susceptibility to thiacetazone should not be viewed as a drug-sensitivity test because of the high concentration of the chemical used. Bonicke (*20*) showed that the compound thiophen-2-carboxylic acid hydrazide was especially useful in differentiating *M. tuberculosis* and *M. bovis,* and his observations were fully confirmed by later studies.

Typing of the Mycobacteria

INTRODUCTION

The first part of this chapter was devoted to an analysis of some procedures used in the identification of mycobacteria to the species level. Now, let us look at other procedures which future investigations may prove to be useful in subdividing species into types. Several investigators use these latter procedures, however, for species identification. At present, the public health significance of typing cannot be assessed; it is also difficult to weigh the usefulness of typing procedures in identification. These procedures fall into three distinct classes: immunological, bacteriophage susceptibility, and bacteriocin susceptibility methods.

Of the immunological methods, four have been used rather extensively. These are: agglutination of bacterial cells by antibodies, precipitin reactions in agar-diffusion techniques, immunofluorescence techniques, and tuberculin testing of sensitized animals. The bacteriophage typing (*135*) and bacteriocin typing, recently suggested by Takeya (*166*), are discussed in Chapter 4.

SEROLOGIC AGGLUTINATION

That the injection of mycobacteria or their constituents in experimental animals elicits the production of antibodies, was shown very early (*14,40,57,70,95,113,130,151*), and various antibodies classified according to their reactions *in vitro* were identified, such as agglutinins, opsonins, precipitins, haemolysins, and complement fixating antibodies. Schaefer (*149*) uses aglutination reactions to analyze the antigenic structure of the mycobacteria. Schaefer's procedure for eliciting antibodies consists of repeatedly injecting rabbits with washed killed bacteria (antigen) by the intravenous route. The agglutination reaction is performed by adding equal volumes of antigen suspension to a series of twofold dilutions of the antiserum and incubating the mixture at 37°C.

A summary of Schaefer's serologic scheme is shown in Tables 7 and 8. *M. kansasii* and *M. marinum* each contain a single serotype. *M. fortuitum* is shown as forming a single serotype and can be differentiated from *M. chelonei (M. abscessus)* by using absorbed serum (*71*).

The study of the incidence of *M. avium-intracellulare* serotypes and *M. scrofulaceum* serotypes in relation to disease in humans and other animals (Tables 8, 9) and the geographic distribution of serotypes (Table 10) is of considerable interest. Although the data now available are still fragmentary, the public health significance of serotyping *M. avium-intracellulare* is apparent. At present, its use is limited by some unresolved discrepancies between serotyping and identification by cultural and biochemical procedures of *M. avium-intracellulare* and *M. scrofulaceum,* by the undecided taxonomic status of *M. intracellulare,* and by the availability of standardized antisera.

IMMUNODIFFUSION PROCEDURES

The application of Ouchterlony (*126,127*) diffusion-in-gel technique to analyze the antigenic composition of the mycobacteria met with some success (*101,102,185,190*). The procedure consists of letting the antigens diffuse from wells into agar containing the antiserums. The results of the antigen-antibody reactions are lines of precipitation, the number and localization of which forms the basis for establishing characteristic precipitation patterns. The same principle was successfully applied by using polyacrylamide gel electrophoresis (*197*).

IMMUNOFLUORESCENCE TECHNIQUES

Immunofluorescence methods have been applied to the mycobacteria (*10, 11, 33, 60, 76, 77, 78*). The wide application of the procedure has been hampered by the unavailability of sera. Furthermore, our experience reveals that cross reactions are difficult to interpret at this time.

TABLE 7. List of Mycobacterial Serotypes[1]

Species	**Serotype**
M. avium-intracellulare	Avium I, II, III, IV, V, VI, VII, Howell, Chance, Altmann, Yandle, Wilson, Watson, Davis, Boone, Darden and Arnold.
M. scrofulaceum	Scrofulaceum-type, Lunning and Gause
M. kansasii	One serotype
M. marinum	One serotype
M. fortuitum	One serotype
M. chelonei (abscessus)	One serotype

[1] The numbered scheme of the serotypes was changed. See Table 8.

TABLE 8. Incidence of *M. avium-intracellulare* and *M. scrofulaceum* Serotypes in Pulmonary and Lymph Node Infections in Man [adapted from Schaefer, *149*]

Serotype		**Lung**	**Lymph Node**	**Total**
Old Designation	**New Designation**			
I	1	30	7	37
II	2	39	15	54
III	3	5	1	6
IV	4	19	3	22
V	5	2	0	2
VI	6	12	10	22
VII	7	14	2	16
Howell	12	15	0	15
Chance	13	11	0	11
Altman	18	9	2	11
Yandle	16	20	5	25
Wilson	17	7	0	7
Watson	9	18	5	23
Davis	8	19	10	29
Boone	14	33	5	38
Darden	19	21	0	21
Arnold	20	8	0	8
M. scrofulaceum	41	5	44	49
Lunning	42	9	33	42
Gause	43	5	11	16
TOTAL	278	301	153	454

TABLE 9. Incidence of *M. avium-intracellulare* and *M. scrofulaceum* Serotypes in Man, Cattle and Swine [adapted from Schaefer, *149*]

Serotype	Man	Cattle	Swine	Chickens and Other Birds
I	37	6	15	22
II	54	42	24	41
III	6	10	5	1
IV	22	8	4	2
V	2	3	3	0
VI	22	1	0	0
VII	16	8	0	0
Howell	15	3	3	1
Chance	11	0	0	0
Altmann	11	0	2	0
Yandle	25	2	0	0
Wilson	7	0	0	0
Watson	23	5	6	0
Davis	29	9	12	0
Boone	38	1	1	0
Darden	21	0	0	0
Arnold	8	0	1	0
M. scrofulaceum	49	0	4	0
Lunning	42	0	3	0
Gause	16	0	0	0
TOTAL	454	98	83	67

TABLE 10. Geographic Distribution of *M. avium* Serotypes in Human (Incidence by Country) [adapted from Schaefer, *149*]

Country	Strains	Type I	Type II
North America	297	17	12
Netherlands (lymph node)	30	2	0
Western Australia	30	1	1
Wales	70	9	21
Denmark	24	1	8
Czechoslovakia	23	1	7

TUBERCULIN TESTING

Two phenomena, described by Robert Koch in 1890, mark the beginning of immunology studies in the mycobacterioses. Koch wrote (*83*): "If a normal guinea pig is inoculated with a pure culture of tubercle bacilli, the wound, as a rule, closes and in the first few days seemingly heals. After ten to fourteen days, however, there appears a firm nodule which soon opens, forming an ulcer that persists until the animal dies. Quite different is the result if a tuberculosis guinea pig is inoculated with tubercle bacilli. For this purpose, it is best to use animals that have been infected four to six weeks previously. In such animals, also, the little inoculation wound closes at first, but in this case no nodule is formed. On the next or second day, however, a peculiar change occurs at the inoculation site. The area becomes indurated and assumes a dark color, and these changes do not remain limited to the inoculating point, but spread to involve an area 0.5 to 1.0 cm in diameter. In the succeeding days, it becomes evident that the altered skin is necrotic. It finally sloughs, leaving a shallow ulcer which usually heals quickly and permanently, and the regional lymph nodes do not become infected. The action of tubercle bacilli upon the skin of a normal animal is thus entirely different from their action upon the skin of a tuberculous one. This striking effect is produced not only by living tubercle bacilli, but also by dead bacilli, whether killed by prolonged low temperature, by boiling, or by certain chemicals."

One will note that this phenomenon, known as Koch's phenomenon, can be resolved into three components: an accelerated inflammatory reaction, the healing of the ulcer, and the failure of the regional lymph nodes to become enlarged as the result of the infection. The first is indicative of hypersensitivity (allergic reaction), whereas the latter components are indicative of acquired resistance. A discussion on the role of hypersensitivity in the accelerated tubercle formation and in tuberculosis immunity is not within the scope of this book. The reader may find such discussions in other books or monographs (*28,41,105,107.140,143*).

The hypersensitivity or the allergic reaction can be demonstrated by injecting a small amount of a concentrated cell-free filtrate of autoclaved cultures of the tubercle bacilli in the host animal. Such filtrates were called tuberculin by Koch (Koch's Old Tuberculin). When injected intracutaneously in an infected animal, the filtrate elicits at the injection site an inflammatory reaction that reaches its peak in 24 to 48 hours. This reaction is called the tuberculin reaction. Extensive investigations on the nature of the components of Old Tuberculin that were responsible for the reaction showed them to be proteins (*103,152,153*). The active proteins were partially purified by precipitation with trichloroacetic acid and were designated PPD (purified protein derivative). The original PPD contained considerable amounts of polysaccharides and nucleic acids. Another more purified product was obtained by ultra-filtration followed by precipitation with ammonium sulfate (*69*). A product prepared in this manner by Florence Seibert, was chosen as a standard, and it is designated PPD-S (for standard PPD). The PPD-S is officially deposited in the Statens Serum Institute in Copenhagen, Denmark; in the United States, it is deposited in the Division of Biologics Standards of the National Institutes of Health, Bethesda, Maryland, and in the Mycobacterial Immunology Section, Mycobacteriology Branch of the Center for Disease Control, Atlanta, Georgia. All newly manufactured tuberculins must be assayed for potency against PPD-S. One tuberculin unit (TU) is defined as the amount of tuberculin that elicits a reaction equivalent to the reaction elicited by 0.00002 mg of PPD-S.

Affronti (*2*) prepared antigens from two strains of *M. kansasii* isolated by Pollak and Buhler, from two strains of *M. intracellulare* isolated from patients in Battey Hospital, Rome, Georgia, and a strain of *Nocardia asteroides.* These antigens were prepared by Seibert's method, and these PPD's were shown to elicit a reaction in all sensitized animals, but in rabbits injected with each of the organisms the degree of skin sensitivity was much greater to the homologous PPD. Further studies confirmed the notion that infected animals react more strongly with the antigens homologous to the infecting organism (Fig. 8), and these observations serve as the basis for using skin testing for identification purposes (*30,45,46,108,109,110*). Antigens available at CDC are listed in Table 11. The use of these products in the diagnosis of mycobacterial infection in man is discussed in Part 2.

TABLE 11. PPD Skin Test Antigens Available at CDC

PPD	Species	Strain
PPD-S	*M. tuberculosis*	Dorset 49608
PPD-Tuberculin	*M. tuberculosis*	DT-612
PPD-Y	*M. kansasii*	Bostron 035
PPD-P	*M. marinum* (syn. *M. platypoecilus*)	Platy 613
PPD-G	*M. scrofulaceum*	Gause 210
PPD-B	*M. intracellulare*	Boone 100616
PPD-F	*M. fortuitum*	Martin 383
PPD-Cleveland Glaze	*M. fortuitum*	Glaze 594

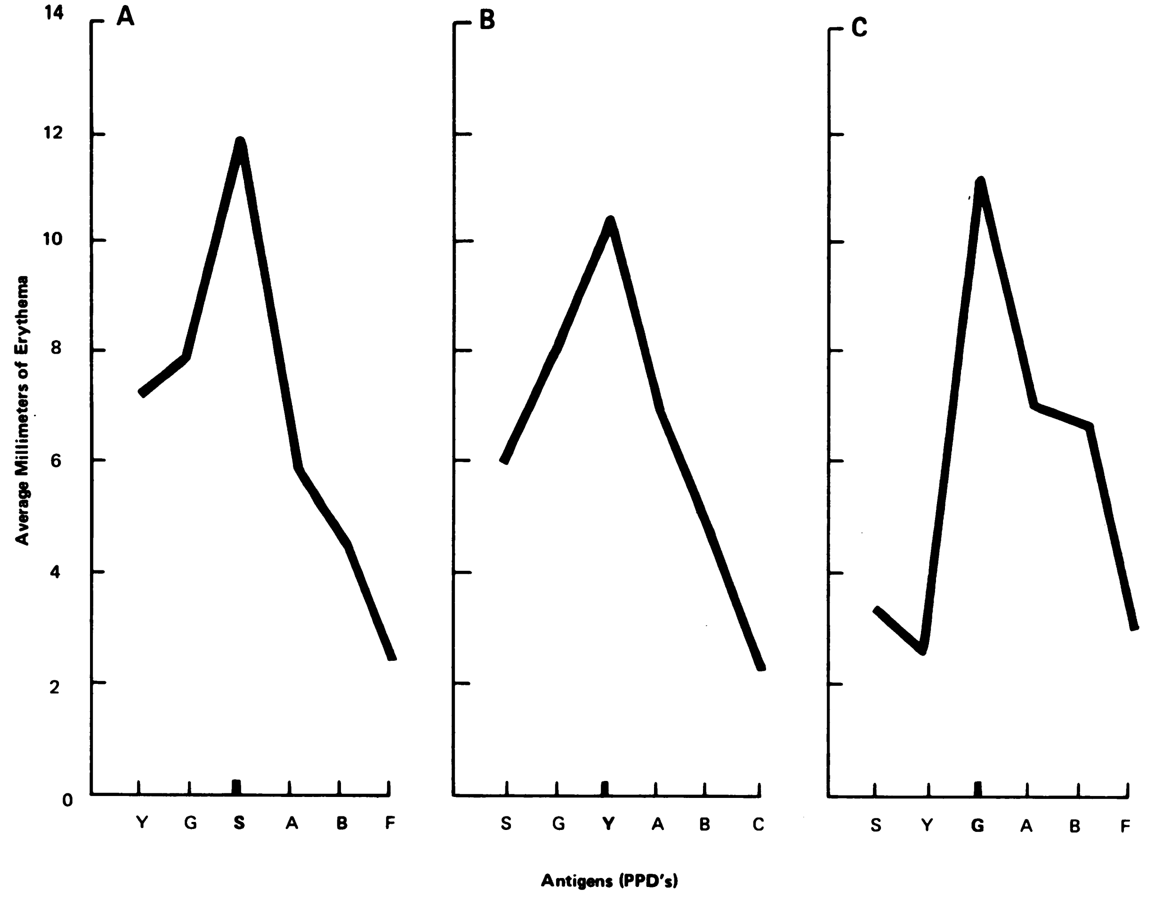


FIGURE 8. Sensitivity profiles to homologous and heterologous PPD's in guinea pigs (adapted in a modified form from Edwards *et al.*, *46*). A—animals sensitized with *M. tuberculosis*; B—animals sensitized with *M. kansasii*; C—animals sensitized with *M. scrofulaceum.*

Retrospective View

Genetic evidence reviewed in Part 1, Chapter 4, indicates that mutations occur in the mycobacteria at frequencies comparable to those for other bacteria. Therefore, standardized procedures to determine the genetic attributes of mycobacterial species give reliable and reproducible results. Various investigators, working independently or in wide-scale cooperative projects, have confirmed this view by applying methods of numerical taxonomy.

In the first part of this review, experimental conditions that may contribute to variation in test results were emphasized. After Wayne's editorial, "The Mycobacterial Mystique: Deterrent to Taxonomy," appeared (*183*), it became evident that "the normally occurring dissociants or mutants of mycobacteria show a low frequency of occurrence, and the forms on which our classifications are based are quite stable if handled and tested with standardized procedures." (*88*).

Fourteen procedures found to be of diagnostic value in the identification of the mycobacteria commonly isolated in clinical laboratories are listed in Table 3. The standard ways to perform them are described on pages 8-10, 18, 26-34. The diagnostic tests described represent a by-product of taxonomic investigations, whose object is to group bacteria having similar properties and to establish the natural relationships between them. Finding overall similarities and determining phylogenetic relationships have been the two major objectives of students of mycobacterial taxonomy. To find the overall similarity between strains of bacteria, numerical taxonomic methods of analysis were adopted. Sokal and Sneath (*162*) define numerical toxonomy as "numerical evaluation of the affinity or similarity between taxonomic units and the ordering of these units into taxa on the basis of their affinity." The problem consists of finding the properties of the strains by using a large number of distinct tests and in finding the similarity between strains expressed as the ratio of characters in common to the total number of characters compared, usually expressed as a percentage. A recent international cooperative study (*191*) on the scotochromogenic slow-growing mycobacteria may serve to exemplify the principles involved. Sixty carefully checked cultures were sent for testing to 11 laboratories in the United States, Canada, Europe, and Japan. Each investigator used the tests he wanted to, and the end result was that many characters were coded for each strain. After irrelevant (there were data identical to all organisms in the study) and repetitious data were deleted, 140 characters were selected and employed in the preparation of a similarity matrix of matches by highest single link, with negative matches included. Presentation of the data in this manner makes the relationships of the strains immediately obvious, strains with high overall similarity appearing as clusters (Fig. 9). Seven clusters were identified; they consisted of: Cluster 1, *M. flavescens*; Cluster 2, *M. gordonae;* Cluster 3, no species designation proposed; Cluster 4, *M. scrofulaceum;* Cluster 5, two strains of *M. intracellulare* are used for comparison and a strain listed in the ATCC catalog as *M. scrofulaceum* ATCC 19073; Cluster 6, two strains of *M. xenopi*; and Cluster 7, a poorly related set of three cultures of unclear taxonomic position.

Among the strains investigated, some were unrelated to the subject of the investigation (a study on the scotochromogenic mycobacteria) and were included for comparative purposes, or as controls.

Once the analysis is done, the investigator can determine which characters show the greatest resolving power among clusters and the expected frequency or probability of a strain belonging to one species sharing a character most often found in strains from other species. These investigations are based on three fundamental principles (*161,162*):

1. All characters are of equal importance.
2. Natural groups should be based on a large number of features.
3. The relationship between groups is a function of the characters being compared.

Runyon (*145*) remarked that "the mycobacterial taxonomist's goal should be the characterization of all mycobacteria in all properties. The valuable by-product, derivation of the best diagnostic properties, is not taxonomy." In this chapter two aspects of the problem were presented, and although the final goal of the taxonomist and of the student of infectious diseases is the same, it so happens that at a certain stage of scientific development their objectives or conceptions may seen divergent to the unattentive observer.

For the sake of completeness, procedures described by various investigators that were not described here are listed in Table 12.

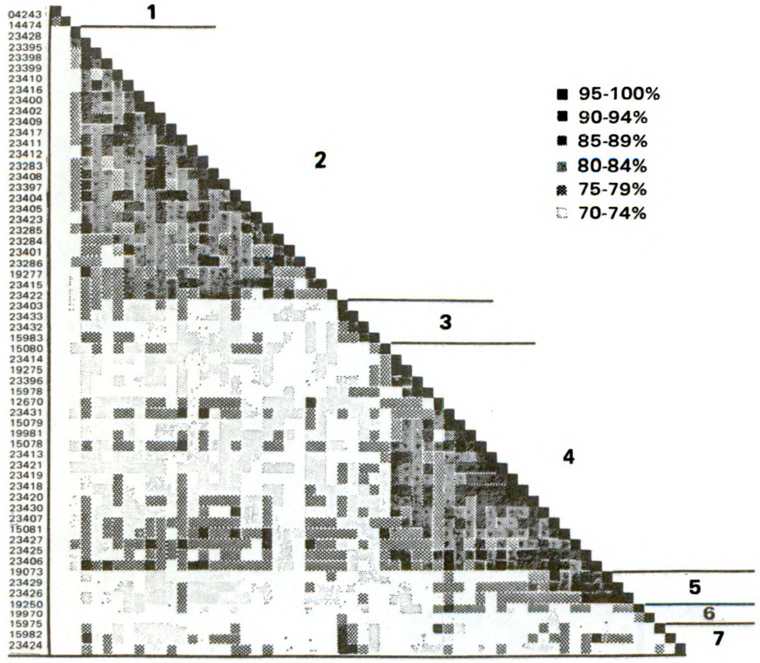


FIGURE 9. Matching matrix of 60 strains of scotochromogenic mycobacteria, based on 140 pooled characters. Note how the matching of the cultures by their similarity showed that they separated into seven clusters at a 75% to 80% level of similarity. Each cluster comprises one species: cluster 1, *M. flavescens;* cluster 2, *M. gordonae;* cluster 3, unnamed; and cluster 4, *M. scrofulaceum.* Clusters 5 and 6 were included as controls and are, respectively, *M. intracellulare* and *M. xenopi* cultures. Culture 7 is a group of three strains of unclear taxonomic position (adapted from Wayne *et al., 191*).

TABLE 12. Procedures Used or Proposed by Various Investigators for the Identification of the Mycobacteria
[The procedures analyzed in the text are not included in this table.]

Procedure	References
Peptidase activity with chromogenic substrate	116
Transformation of ferric salts;	174
Accumulation of iron in the cells	188
Pepitase activity	131
Tolerance to picric acid	176
Inhibition by neotetrazolium	59
Amidase Activity	18,21
Inhibition by 8-oxyquinoline	165
Electrophoretic behavior of esterases and catalases	122,123
Mycosides (species specific glycolipids)	31,32,59,99,125,134 157,158,159
Thin layer chromatography of lipids	136
Pyrolysis gas liquid chromatography	136,137,138,139
Susceptibility to hydroxylamine and 8-azaguanine	176
Susceptibility to dyes	72
Inhibition by p-Nitro-2-acetylamino-β-hydroxy-propiophenone	47
Urease activity	172
Pyrazinamidase activity	186
Hemolysis of guinea-pig erythrocytes	167
Acid phosphatases	5
Formamidase activity	121
Cultural and physiological properties	54,61,62,63,154,170

Laboratory Procedures

NIACIN TEST

•Test Tube Procedure (*84,147*)

Equipment

Sterile screwcap test tubes, 16 x 125 mm

Reagents

☐ *Sterile distilled water or 85% saline*

☐ *4% aniline solution*

To 96.0 ml of 95% ethanol, add 4.0 ml of colorless aniline. Store in a brown bottle in the refrigerator. If solution turns yellow, discard.

☐ *10% cyanogen bromide*

Dissolve 5.0 g cyanogen bromide in 50 ml of distilled water. Store in a tightly capped brown bottle in the refrigerator. If precipitate forms upon cooling, warm to room temperature to dissolve.

Procedure

To the culture slant on Lowenstein-Jensen medium at least 4 weeks old, add 1.0 ml of sterile distilled water or saline. If the culture is confluent, break the cell mass to facilitate extraction from the medium (most niacin is extra-cellular and accumulates in the medium).

Allow the extraction to proceed for at least 15 minutes. Place the tube slanted so that the fluid covers the entire surface of the medium.

Remove 0.5 ml of the fluid extract and transfer to a screwcap tube.

Add 0.5 ml of the aniline solution. This should be colorless.

Then, add 0.5 ml of the cyanogen bromide solution.

Observe for the formulation of a yellow color, which should develop immediately.

Safety

Cyanogen bromide (tear gas) is very toxic and, in contact with acids, is converted to hydrocyanic acid. The compound must, therefore, be handled in a well-ventilated safety cabinet. The test tubes used in performing the tests must be discarded into a germicidal solution made alkaline by the addition of sodium hydroxide.

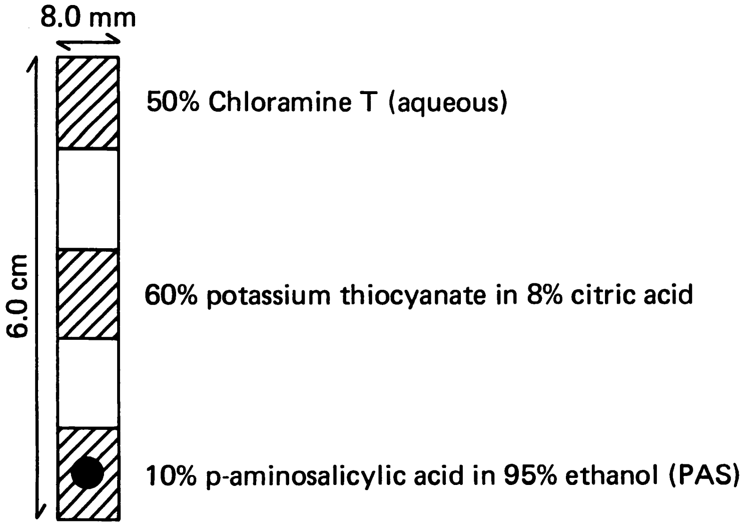
Quality controls

Cyanogen bromide is volatile. Because weak solutions give false negative results, the reagent must be prepared in small quantities.

Reagent controls must be performed on extracts from uninoculated medium, on extracts from a known culture of *M. intracellulare* (negative control), and on extracts of a culture of *M. tuberculosis* (positive control).

•Paper Strip Procedure

These strips are commercially available. As described by Kilburn and Kubica (*84*), the strips are 6.0 cm long by 8.0 mm wide, and the reagents are impregnated into the paper as shown below:


8.0 mm
6.0 cm
50% Chloramine T (aqueous)
60% potassium thiocyanate in 8% citric acid
10% p-aminosalicylic acid in 95% ethanol (PAS)

Procedure

To 0.5-0.6 ml of the extract obtained as described above, insert the strip, PAS end first, in the test tube containing the extract. Stopper at once.

Shake gently to facilitate diffusion. Do not tilt the tube.

Repeat shaking after 5 or 10 minutes.

After 15 minutes, but no later than 30 minutes, compare the color against a white background.

Quality controls

Same as indicated for the test tube procedure.

Note: Niacin test must not be performed in 7H10 medium unless the medium is supplemented with 0.1% of potassium aspartate (*81*).

NITRATE REDUCTION (*180*)

•Equipment

Screwcap test tubes, 16 x 125 mm.

Sterile pipettes, capillary or 1.0 and 5.0 ml.

Inoculating spade or loop.

Water bath at 37°C, or constant temperature block heater.

•Reagents

Substrate

0.01 M sodium nitrate in 1/45 M phosphate buffer, pH 7.0.

$NaNo_3$	0.85 g
KH_2PO_4	0.117 g
$Na_2HPO_4 \bullet 12 H_2O$	0.485 g
Distilled Water	100.0 ml

Reagent #1 – a 1:2 dilution of HCl in water (10.0 ml of HCl plus 10.0 ml of water).

Reagent #2 – 0.2 mg sulfanilamide in 100.0 ml of distilled water.

Reagent #2 – 0.1 g of N-naphtylenediamine dihydrochloride in 100.0 ml of distilled water.

Store the substrate and reagents in the dark in the refrigerator. If precipitate forms or the reagents change color, discard.

•Procedures

Place three or four drops of distilled water in a sterile screwcap test tube.

Emulsify in the water one loopful or spadeful of the bacterial mass from a solid medium.

Add 2.0 ml of the $NaNO_3$ substrate solution.

Shake by hand to mix and then incubate at 37°C for 2 hours.

Remove from the temperature bath.

Add one drop of reagent #1.

Add one drop of reagent #2.

Add one drop of reagent #3.

Observe for the formation of a red color:

If a red color develops, the test is positive. The color intensity may range from pink (±) to deep red (5+), by comparison with the color standards described below.

If no color develops, the test is either negative or the enzymatic reduction has proceeded beyond nitrite. For confirmation, add a small amount of powdered zinc to the reaction mixture. The powdered zinc catalytically reduces nitrate into nitrite, and therefore a truly negative test becomes red when the catalyst is added.

•Color Standard

Stock solutions

M/15 disodium phosphate. In a volumetric flask dissolve 9.47 g of anhydrous Na_2HPO_4 in distilled water to make 1,000 ml.

M/15 monopotassium phosphate. In a volumetric flask dissolve 9.07 g of KH_2PO_4 in distilled water to make 1,000 ml.

M/15 trisodium phosphate. In a volumetric flask dissolve 25.33 g of $Na_3PO_4 \bullet 12\ H_2O$ in distilled water to make 1,000 ml.

1% phenolphtalein. Dissolve 1.0 g in 100.0 ml of 95% ethanol.

1% bromothymol blue. Dissolve 1.0 g in 100.0 ml of 95% ethanol. To make a 0.01% solution, add 1.0 ml of the 1% solution to 100.0 ml of distilled water.

Working buffer solution

Stock #1	35.0 ml
Stock #2	5.0 ml
Stock #3	100.0 ml

Preparation of standards

1. Line up eight clean test tubes in a rack.
2. Distribute 2.0 ml of working buffer solution into seven tubes.
3. To 10.0 ml of working buffer solution, add 0.1 ml of 1% phenolphtelein and 0.2 ml of 0.01% bromothymol blue.

4. Add 2.0 ml of solution in step 3 above to the eighth tube. This is the 5+ color standard.
5. To the first tube in the series of seven tubes (step 2) add 2.0 ml of solution prepared in step 3. Mix well. This is the 4+ color standard.
6. Carry over 2.0 ml of the 4+ color standard to the next tube in the series; continue to make twofold serial dilutions, discarding 2.0 ml from the last seventh tube.
   The color scale is: tube in step 4, 5+; remaining tubes in order of decreasing intensity, 3+; 2+; 1+, and ±.
7. Autoclave the tubes, seal, and store in the refrigerator.

•Quality Control

The cultures must be 3 to 4 weeks old.

Reagent controls

Sterile system with addition of zinc powder (positive result).

Sterile system without the addition of zinc powder (negative result).

Glassware must be thoroughly washed and cleaned.

Note: For the purpose of establishing the identification tables, a positive result is a reaction of at least 3+.

CATALASE TESTS

•Equipment

Pipettes, capillary or 1.0 ml and 5.0 ml.

Screwcap test tubes.

Water bath or constant temperature block heater.

Inoculating spade or loop.

•Reagents

30% hydrogen peroxide (Superoxol)
Store in refrigerator

10% Tween 80
Autoclave for 10 minutes at 121°C and store in refrigerator.

•Substrate

Immediately before testing, mix equal volumes of Superoxol and the Tween 80 solution.

M/15 phosphate buffer
pH 7.0: mix 61.1 ml of M/15 disodium phosphate (9.47 g of Na_2HPO_4 in 1,000 ml distilled water) with 38.9 ml of M/15 monopotassium phosphate (9.07 g of KH_2PO_4 in 1,000 ml of distilled water).

•Procedure

Room temperature test (114)

Add one or two drops of the Tween-hydrogen peroxide mixture to the culture growth on solid medium. Observe for the formation of bubbles, which may require up to 5 minutes.

pH 68°C Test (*89*)

With a sterile pipette dispense 0.5 ml of M/15 phosphate buffer, pH 7.0, into a screwcap test tube.

With a loop or spade emulsify the bacterial mass from solid medium into the buffer.

Place the tube in a water bath or temperature block heater at 68°C for 20 minutes (the time and temperature are critical).

Remove the tubes and cool to room temperature.

Add 0.5 ml of the Tween peroxide mixture.

Observe for the formation of bubbles appearing at the surface of the liquid. Hold the tubes for 20 minutes before reporting a negative result.

Semiquantitative catalase test (*182*)

☐ *Preparation of cultures*

Dispense 5.0 ml of liquid Lowenstein-Jensen medium into sterile 18 x 150 mm screwcap test tubes.

Inspissate the medium in an upright position to form a butt of solid medium. This can be done in a water bath adjusted to 86°C.

Inoculate the surface of the medium with 0.1 ml or using a 3.0 mm loop of a 7-day-old culture in liquid medium.

Incubate at 37°C with the caps loose for 2 weeks.

☐ *Procedure*

Add 1.0 ml of the Tween-hydrogen peroxide mixture.

Leave the tube in an upright position at room temperature for 5 minutes.

Measure in millimeters the height of the column of bubbles above the surface of the medium.

Record the results as follows:
Negative = no bubbles
Less than 45 mm = low catalase
More than 45 mm = high catalase

ARYLSULFATASE TEST *(90,92)*

•Substrate

Dissolve 2.6 g tri-potassium phenolphtalein disulfate in 50.0 ml of distilled water (0.08 M solution).

Sterilize by filtration.

Store in the refrigerator.

•Medium

Prepare 200.0 ml of Dubos broth medium. The medium is commercially available; prepare according to the manufacturer's instructions.

For the 3-day test, add 2.5 ml of the 0.08 M solution of the substrate to the broth.

For the 2-week test, add 7.5 ml of the 0.08 M solution of the substrate to the broth and dispense aseptically in 2.0 ml amounts in 16 x 135 mm screwcap tubes.

•Reagent

2.0 N sodium carbonate: dissolve 10.6 g of anhydrous Na_2CO_3 in 100.0 ml of distilled water.

•Procedure

Inoculate one tube of each with 0.1 ml of a 7-day liquid culture or a spadeful of organisms from a freshly grown culture.

Incubate at 37°C.

After 3 days of incubation, remove the 3-day tube from the incubator and add no more than six drops of 2 N Na_2CO_3 solution; after 2 weeks remove the other tube and do the same.

Result – The development of a pink or red color indicates a positive test result. Record the intensity of color against a standard prepared as described below.

•Color Standard

M/15 disodium phosphate.

0.1% stock solution of phenol red: 0.1 g of phenolsulfonphtalein sodium salt in 100.0 ml of distilled water. Autoclave and store in the refrigerator.

Prepare the color standard in 16 x 125 mm screwcap tubes as follows:

Tube #	*M/15 Na_2HPO_4*	*0.1% Phenol red*	*Reading*
1	4.0 ml	10 drops	5+
2	4.0 ml	1.0 ml from tube #1	4+
3	3.0 ml	2.0 ml from tube #2	3+
4	3.0 ml	2.0 ml from tube #3	2+
5	3.0 ml	2.0 ml from tube #4	1+
6	3.0 ml	2.0 ml from tube #5	±

Discard 2.0 ml from tube #6. Autoclave, seal, and store in refrigerator.

The standard retains its color for about 6 months.

•Quality Controls

Free phenolphthalein in the substrate

When free phenolphthlein is present in the substrate, uninoculated medium may become pink or red when Na_2CO_3 solution is added. If this happens, recrystallize with absolute ethanol (free Phenolphtalein is soluble in ethanol; tri-potassium phenolphthalein disulfate is insoluble in ethanol).

Test controls

Uninoculated tube of medium (negative).

Medium inoculated with *M. fortuitum* (positive).

TWEEN 80 HYDROLYSIS (*181,189*)

•Substrate

Combine 100.0 ml M/15 phosphate buffer pH 7.0, 0.5 ml Tween 80, and 2.0 ml of a 0.1% aqueous neutral red stock solution. The Tween 80 must be diluted in buffer before the neutral red solution is added.

Dispense in 2.0 ml amounts into 16 x 125 mm screwcap tubes.

Autoclave 10 minutes at 121°C. After autoclaving, the liquid should be amber colored.

The substrate may be stored in the dark in the refrigerator for no more than 2 weeks.

•Procedure

Suspend in the substrate solution a 3-mm loopful of organisms from solid medium.

Incubate at 37°C.

•Results

The development of a pink to red color in the liquid indicates a positive test result.

Read the tubes at 24-hour, 5-day, and 10-day intervals. Do not shake the tubes when reading. Record the results, and discard the positive tubes at each time interval.

•Quality Controls

M. kansasii (positive).

Uninoculated substrate (negative).

TELLURITE REDUCTION (*82*)

•Materials

Medium

Middlebrook 7H9 medium (5.0 ml in 18 x 150 mm screwcap tubes).

Reagent

0.2% solution of potassium tellurite sterilized by autoclaving.

•Procedure

Inoculate the liquid medium with the culture to be tested, using a heavy inoculum.

Incubate at 35-37°C for 7 days.

With a capillary pipette add two drops of the potassium tellurite solution.

Reincubate at 35-37°C for an additional 3 days.

Results

Do not shake the tubes. Observe the color of the cell mass deposited at the bottom of the tube. A black metallic precipitate indicates a positive test result.

Quality Controls

M. avium-intracellulare (positive).

Uninoculated medium (negative).

REFERENCES

1. Abbott, V. D., Kilburn, J. O., Harbison, W., and Kubica, G. P. (1968) Amer. Rev. Resp. Dis. 97:931.
2. Affronti, L. F. (1958) Amer. Rev. Resp. Dis. 79:284.
3. Albertson, J. N., and Moat, A. G. (1965) J. Bacteriol. 89:540.
4. Anderson, R. J. (1932) Physiol. Rev. 12:166.
5. Andrejew, A. and Renard, A. (1968) Ann. Inst. Pasteur 115:343.
6. Andrejew, A. and Gernez-Rieux, C., and Tacquet, A. (1960) Ann. Inst. Pasteur (Paris) 99:56.
7. Baess, I., and Weiz-Bentson, M. (1969) Acta Pathol. Microbiol. Scandinavica 75:731.
8. Bastarrachea, F., and Goldman, D. S. (1961) Biochem. Biophys. Acta 50:174.
9. Bennedsen, J. (1966) Acta Pathol. Microbiol. Scand. 68:262.
10. Bennedsen, J. (1968) Acta Pathol. Microbiol. Scand. 72:330.
11. Bennedsen, J. (1969) Acta Pathol. Microbiol. Scand. 76:245.
12. Bergey's Manual of Determinative Bacteriology, 7th Edition, The Williams and Wilkins Co., 1957, Baltimore, Md.

12a. Idem, 8th edition, 1974.

13. Birn, K., Schaefer, W. B., Jenkins, P. A., Szulga, T. and Marks, J. (1967) J. Hyg. 65:575.
14. Boquet, A. and Nègre, L. (1922) Compte r. Soc. Biol. 86:581.
15. Bogen, E. (1941) Amer. Rev. Tuberc. 44:267.
16. Boisvert, H. (1967) Rassegna di Patologia del Apparato Respiratorio, 1967.
17. Bojalil, L. F., Cerbón, J. and Trujillo, A. (1962) J. Gen. Microbiol. 28:333.
18. Bönicke, R. (1957) Zbl. Bakt. 170:355.
19. Bönicke, R. (1958) Zschr. Hyg. 145:263.
20. Bönicke, R. (1958) Naturwiss 45:392.
21. Bönicke, R. and Lisboa, B. P. (1959) Zbl. Bakt. 175:403.
22. Bönicke, R. and Juhasz, S. E. (1964) Zbl. Bakt. 192:133.
23. Bönicke, R. and Stottmeier, D. (1965) Beitr. Klin. Tuberk. 130:210.
24. Bönicke, R. and Kazda, J. (1970) Zentralbt. Bakteriol. Infektionsk. Hyg. 213:68.
25. Bönicke, R. and Juhasz, S. (1965) Zbl. Bakt. 197:292.
26. Bönicke, R., Juhasz, S. E. and Diemer, U. (1970) Amer. Rev. Resp. Dis. 102:507.
27. Brosbe, E. A., Sugihara, P. T., and Smith, C. R. (1962) J. Bacteriol. 84:1282.
28. Canetti, G. (1955) The Tubercle Bacilli in the Pulmonary Lesion of Man, Springer Pub. Co., Inc., N.Y., 1955.
29. Cerbón, J. and Bojalil, L. F. (1961) J. Gen. Microbiol. 25:7.
30. Chaparas, S. D., Maloney, C. J. and Hedrick, S. R. (1970) Amer. Rev. Resp. Dis. 101:74.
31. Chaput, M., Michele, G. and Lederer, E. (1962) Biochem. Biophys. Acta 63:310.
32. Chaput, M., Michele, G. and Lederer, E. (1963) Biochem. Biophys. Acta 78:329.
33. Choi, C. S., Royal, W. A. and Francis, J. (1971) Tubercle 52:148.
34. Clancey, J. K. (1964) J. Pathol. Bacteriol. 88:175.
35. Clark, H. F. and Shepard, C. C. (1963) J. Bacteriol. 86:1057.
36. Collins, C. H. (1966) J. Clin. Pathol. 19:433.
37. Davis, B. D. and Dubos, R. J. (1948) J. Bacteriol. 55:11.
38. DeTurk, W. E. and Bernheim, F. (1958) J. Bacteriol. 75:691.
39. DiSalvo, A. F. and Lindler, G. N. (1970) Amer. J. Clin. Pathol. 53:871.
40. Doan, C. A. (1929) Proc. Soc. Expt. Biol. Med. 26:672.
41. Dubos, R. J. (1954) Biochemical determinants of microbiol. diseases, Harvard University Press, Cambridge, 1954.
42. Dudley, M. A. and Willet, H. P. (1965) Proc. Soc. Exp. Biol. 119:807.
43. Dudley, M. A. and Willet, H. P. (1966) Canadian J. Microbiol. 12:1225.
44. Edson, N. L. (1951) Bacteriol. Rev. 15:147.
45. Edwards, L. B., Palmer, C. E., Affronti, L. F., Hopwood, L. and Edwards, P. Q. (1960) Am. J. Hyg. 71:218.
46. Edwards, L., Hopwood, L., Affronti, L. F. and Palmer, C. E. (1962) Bull. Int. Union Tub. 32:384.
47. Eidus, L., Diena, B. B., Greenberg, L. (1960) Amer. Rev. Resp. Dis. 81:759.
48. Engbaek, H. C., Vergmann, B., Baess, I. and Will, D. W. (1967) Acta Pathol. Microbiol. Scand. 69:576.
49. Engbaek, H. C., Vergmann, B., Baess, I., and Weiz-Bentson, J. (1968) Acta Pathol. Microbiol. Scandinavica 72:277.

50. Engbaek, H. C., Runyon, E. H. and Karlson, A. G. (1971) Int. J. Syst. Bacteriol. 21:192.
51. Feige, F. (1966) Spot Tests in Organic Chemistry, 7th edition, p. 384-386, Elsevier Publishing Co., New York.
52. Feige, A., Gross, D., Zureck, A. and Schutte, H. R. (1970) Arch. Mikrobiol. 75:80.
53. Fields, B. T., Bishop, M. C., Brosbe, E. A. and Bates, J. H. (1969) Amer. Rev. Resp. Dis. 99:590.
54. Forey, C. A. and Hajan, W. A. (1931) J. Infect. Dis. 49:497.
55. Fregnan, G. B., Smith, D. W. and Randall, H. M. (1961) J. Bacteriol. 82:517.
56. Fregnan, G. B. and Smith, D. W. (1962) J. Bacteriol. 83:828.
57. Furth, J. (1926) J. Immunol. 12:273.
58. Gangadharam, P. R. G. and Droubi, A. (1971) Amer. Rev. Resp. Dis. 104:434.
59. Gastambide-Odier, M. and Smith, D. W. (1958) Amer. Rev. Tuberc. 77:662.
60. Gillissen, G. (1963) Zbl. Bakt. 188:81.
61. Gordon. R. E. (1937) J. Bacteriol. 34:617.
62. Gordon, R. E. and Mihm, J. M. (1959) J. Gen. Microbiol. 21:736.
63. Gordon, R. and Smith, M. M. (1955) J. Bacteriol. 69:502.
64. Gosio, B. (1905) Ztschr. Hyg. 51:65.
65. Hauduroy, P., Bel, F. and Havanessian, A. (1966) Ann. Inst. Pasteur (Paris) 111:82.
66. Hauduroy, P. (1955) Derniers aspects du Monde der Mycobacteries, Masson et Cie., Paris, 1955.
67. Hedgecock, L. W. and Costello, R. L. (1962) J. Bacteriol. 84:195.
68. Hobby, G. *et al* (1964) Amer. Rev. Resp. Dis. 89:337.
69. Holm, J. and Lind, P. (1947) Pub. Health Rep. 62:188.
70. Hughes, J. (1933) J. Immunol. 25:103.
71. Jenkins, P. A., Marks, J. and Schaefer, W. B. (1971) Amer. Rev. Resp. Dis. 103:179.
72. Jones, W. D., Jr. and Kubica, G. P. (1963) Amer. Rev. Resp. Dis. 88:355.
73. Jones, W. D., Jr. and Kubica, G. P. (1964) Amer. J. Med. Technol. 30:187.
74. Jones, W. D., Jr. and Kubica, G. P. (1965) Amer. Rev. Resp. Dis. 91:613.
75. Jones, W. D., Jr. and Kubica, G. P. (1965) Zentralb. Bakteriol. Parasit. Infektionk. und Hyg. 196:68.
76. Jones, W. D., Jr., Saito, H. and Kubica, G. P. (1965) Amer. Rev. Resp. Dis. 92:255.
77. Jones, W. D., Jr. and Kubica, G. P. (1968) Zentralb. Bakteriol. Parasit. Infektionk. und Hyg. 207:58.
78. Jones, W. D., Jr., Beam, R. E. and Kubica, G. P. (1967) Amer. Rev. Resp. Dis. 95:516.
79. Karlson, A. G. and Lessel, E. F. (1970) Int. J. Syst. Bacteriol. 20:273.
80. Kilburn, J. O. and Kubica, G. P. (1968) Amer. J. Clin. Pathol. 50:530.
81. Kilburn, J. O., Stottmeier, K. D. and Kubica, G. P. (1968) Amer. J. Clin. Pathol. 50:582.
82. Kilburn, J. O., Silcox, V. A. and Kubica, G. P. (1969) Amer. Rev. Resp. Dis. 99:94.
83. Koch, R. (1890) Deutsch. Med. Worsch. 16:1029.
84. Konno, K. (1956) Science 124:985.
85. Konno, K., Kurzmann, R. and Bind, K. T. (1957) Amer. Rev. Tub. Pulm. Dis. 75:529.
86. Konno, K. and Sbarra, A. J. (1959) Amer. Rev. Tub. Pulm. Dis. 79:810.
87. Konno, K., Oizumi, K. and Oka, S. (1965) Nature 205:874.
88. Kubica, G. P. (1966) Letter to the editor, ARRD. 93:957.
89. Kubica, G. P. and Pool, G. L. (1959) Amer. Rev. Resp. Dis. 81:387.
90. Kubica, G. P. and Vestal, A. L. (1961) Amer. Rev. Resp. Dis. 83:728.
91. Kubica, G. P. and Rigdon, A. L. (1961) Amer. Rev. Resp. Dis. 83:737.
92. Kubica, G. P. and Beam, R. E. (1961) Amer. Rev. Resp. Dis. 83:733.
93. Kubica, G. P. and Jones, W. D., Jr. (1965) Zentralb. Bakteriol. Parasit. Infektionk. und Hyg. 196:53.
94. Kubica, G. P., Silcox, V. A., Kilburn, J. O., Smithwick, R., Beam, R. E., Jones, W. D., Jr. and Stottmeier, K. D. (1970) Int. J. Syst. Bacteriol. 20:161.
95. Laidlaw, P. P. and Dudley, H. W. (1925) Brit. J. Exp. Path. 6:197.
96. Lambe, D. W. and Kubica, G. P. (1962) Amer. Rev. Resp. Dis. 86:117 (abstract).
97. Laneele, G. and Asselineau, J. (1962) Biochem. Biophys. Acta 59:731.
98. Laporte, R. and Loiseleur, J. (1945) Ann. Inst. Pasteur (Paris) 71:375.
99. Lederer, E. (1967) Chemistry and Physics of Lipids 1:294.
100. Lenel, E. F. (1967) Int. J. Syst. Bacteriol. 17:241.
101. Lind, A. (1961) Acta Pathol. Microbiol. Scand. 144:51.

102. Lind, A. and Norlin, M. (1963) Scand. J. Clin. Lab. Invest. Suppl. 69, p. 152.
103. Long, E. R. and Seibert, F. B. (1926) Amer. Rev. Tub. 13:448.
104. Lorian, V. (1969) Appl. Microbiol. 17:559.
105. Lurie, M. (1964) Resistance to tuberculosis; experimental studies in active and acquired defensive mechanisms. Harvard University Press, Cambridge, 1964.
106. MacCallum, P., Tolhurst, J. C., Buckle, G. and Sisson, H. A. (1948) J. Pathol. and Bacteriol. 60:93.
107. Mackaness, G. (1971) J. Infect. Dis. 123:439.
108. Magnusson, M. (1961) Amer. Rev. Resp. Dis. 83:57.
109. Magnusson, M. (1961) Amer. Rev. Resp. Dis. 83:69.
110. Magnusson, M. (1967) Zeitsch. Tuberk. und Erkrankungen Toraxorg. 127:55.
111. Magnusson, M., Engbaek, H. C. and Bentzon, M. (1961) Amer. Rev. Resp. Dis. 83:69.
112. Martins, R. R., Walker, W. E., Batayias, G. E. and Gales, P. W. (1973) Amer. Rev. Resp. Dis. 108:979.
113. McCutcheon, M., Strumia, M., Mudd, S., Mudd, E. and Lucke, B. (1929) J. Expt. Med. 49:815.
114. Middlebrook, G. (1954) Amer. Rev. Tub. 64:471.
115. Mudd, S., Takeya, K. and Henderson, H. J. (1956) J. Bacteriol. 72:767.
116. Muftic, M. (1967) Folia Microbiol. 12:500.
117. Murohashi, T. and Yoshida, K. (1965) Amer. Rev. Resp. Dis. 92:817.
118. Murohashi, T. and Yoshida, K. (1966) Amer. Rev. Resp. Dis. 94:86.
119. Murohashi, T., Yoshida, K. and Kato, M. (1966) Amer. Rev. Resp. Dis. 94:114.
120. Murohashi, T. and Yoshida, K. (1968) Amer. Rev. Resp. Dis. 97:306.
121. Nagayama, H., Konno, K. and Oka, S. (1961) Nature 190:1279.
122. Nakayama, Y. (1967) Japan. J. Microbiol. 11:95.
123. Nakayama, Y. and Takeya, K. (1967) Nature 213:504.
124. Nassau, E. and Merrick, A. J. (1970) Tubercle 51:430.
125. Navalkar, R., Wiegeshaus, E., Kondo, E., Kim, H. and Smith, D. (1965) J. Bacteriol. 90:262.
126. Ouchterlony, O. (1958) Progr. Allergy 5:1.
127. Ouchterlony, O. (1962) Progr. Allergy 6:30.
128. Pattyn, S. R. (1966) Zentralb. Bakteriol. Parasitenk. Infektionk. und Hyg. 201:246.
129. Pattyn, S. R. (1967) Zeitschrift Tuberk. 127:41.
130. Pinner, M. (1928) Amer. Rev. Tuberc. 18:497.
131. Plancot, M., Guillermand, J., Roos, P. and Tacquet, A. (1967) Ann. Inst. Past. 113:132.
132. Pope, H. and Smith, D. T. (1946) Amer. Rev. Tuberc. 54:559.
133. Prissick, F. H. and Masson, A. M. (1956) Canadian Med. Assoc. J. 75:798.
134. Randall, H. and Smith, D. (1964) Zbl. Bakt. 194:190.
135. Redmond, W. B. (1963) Adv. Tuberc. Res. 12:191.
136. Reiner, E., Beam, R. E. and Kubica, G. P. (1967) J. Chromat. 27:495.
137. Reiner, E. and Kubica, G. P. (1969) Amer. Rev. Resp. Dis. 99:42.
138. Reiner, E., Beam, R. E. and Kubica, G. P. (1969) Amer. Rev. Resp. Dis. 99:750.
139. Reiner, E., Hicks, J. J., Beam, R. E. and David, H. L. (1971) Amer. Rev. Resp. Dis. 104:656.
140. Rich, A. R. (1951) The Pathogenesis of Tuberculosis, Charles C. Thomas, Publisher, Springfield, Ill. U.S.A., 1951.
141. Richards, O. W., Kline, E. K. and Leach, R. E. (1941) Amer. Rev. Tuberc. 44:255.
142. Rio-Estrada, C. and Patino, H. (1962) J. Bacteriol. 84:871.
143. Rosenthal, S. R. (1957) BCG vaccination against tuberculosis. Little, Brown, Boston, 1957.
144. Runyon, E. (1959) Medical Clinics of North America 43:273.
145. Runyon, E. (1967) Amer. Rev. Resp. Dis. 95:861.
146. Runyon, E. (1968) J. Bacteriol. 95:734.
147. Runyon, E. H., Selin, M. J. and Harris, H. W. (1959) Amer. Rev. Resp. Dis. 79:663.
148. Ryter, A. (1968) Bacteriol. Rev. 32:39.
149. Schaefer, W. B. (1967) Amer. Rev. Resp. Dis. 96:115.
150. Schwabacher, H. (1959) J. Hyg. 57:57.
151. Seibert, F. B. (1928) Amer. Rev. Tub. 17:402.
152. Seibert, F. (1933) Proc. Soc. Exp. Biol. and Med. 30:1274.

153. Seibert, F. B. (1928) Amer. Rev. Tub. 17:402.
154. Shepard, C. C. (1957) J. Bacteriol. 73:722.
155. Sherman, H. (1923) J. Infect. Dis. 12:249.
156. Silcox, V. and David, H. L. (1970) Appl. Microbiol. 21:327.
157. Smith, D., Harrell, W. and Randall, H. (1954) Amer. Rev. Tub. 69:505.
158. Smith, D., Randall, H., Gastambide-Odier, M. and Koevet, A. (1957) Ann. N.Y. Acad. Sc. 69:145.
159. Smith, D., Randall, H., MacLennan, A., and Lederer, E. (1960) Nature 186:887.
160. Smithwick, R. W. and David, H. L. (1971) Tubercle. 52:226.
161. Sneath, P. H. A. (1957) J. Gen. Microbiol. 17:201.
162. Sokal, R. and Sneath, P. H. A. (1963) Principles of numerical taxonomy, W. H. Freeman, San Francisco, 1963.
163. Subcommittee on Mycobacteria, Amer. Soc. for Microbiol. (1962) J. Bacteriol. 83:931.
164. Szabo, I. (1964) Zbl. Bakt. 194:528.
165. Szabo, I. (1966) Nature 212:1384.
166. Takeya, K., and Tokiwa, H. (1972) Int. J. Syst. Bacteriol. 22:178.
167. Takeya, K., Mori, R., Hisatsune, K. and Toda, T. (1963) Amer. Rev. Resp. Dis. 87:773.
168. Tarshis, M. S. (1964) Acta Tub. Pneumol. Scandinavica 45:221.
169. Terai, T., Kamahora, T. and Yamamura, Y. (1958) J. Bacteriol. 75:535.
170. Thompson, H. M. (1932) Amer. Rev. Tub. 26:162.
171. Toda, T., Takeya, K. and Koike, M. (1958) 4th Intern. Congress Electron. Microscopy, Berlin 1958.
172. Toda, T., Hagihara, Y. and Takeya, K. (1961) Amer. Rev. Resp. Dis. 83:757.
173. Truant, J. P., Brett, W. A. and Thomas, W., Jr. (1962) Henry Ford Hospital Medical Bull. 10:287.
174. Tsukamura, M. (1965) Amer. Rev. Resp. Dis. 92:491.
175. Tsukamura, M. (1965) Med. Biol. (Tokyo) 71:110.
176. Tsukamura, M. (1965) J. Bacteriol. 90:556.
177. Tsukamura, M. and Tsukamura, J. (1965) J. Bacteriol. 89:1442.
178. Vestal, A. L. and Kubica, G. P. (1966) Amer. Rev. Resp. Dis. 94:247.
179. Vestal, A. L. and Kubica, G. P. (1967) Scand. J. Resp. Dis. 48:142.
180. Virtanen, S. (1960) Acta Tub. Scandinavica, Suppl. #48.
181. Wayne, L. G. (1962) Amer. Rev. Tub. 86:579.
182. Wayne, L. G. (1962) Amer. Rev. Resp. Dis. 86:651.
183. Wayne, L. G. (1964) Amer. Rev. Resp. Dis. 90:255.
184. Wayne, L. G. (1966) Amer. Rev. Resp. Dis. 93:919.
185. Wayne, L. G. (1971) Infect. Immun. 3:36.
186. Wayne, L. G. (1974) Amer. Rev. Resp. Dis. 109:147.
187. Wayne, L. and Doubek, J. R. (1964) Amer. J. Clin. Pathol. 42:431.
188. Wayne, L. G. and Doubek, J. R. (1968) Appl. Microbial. 16(6):925.
189. Wayne, L. G. and Doubek, J. R. and Russel, R. L. (1964) Amer. Rev. Resp. Dis. 90:588.
190. Wayne, L. G., Doubek, J. R. and Diaz, G. A. (1967) Amer. Rev. Resp. Dis. 96:88.
191. Wayne, L. G. et al. (1971) J. Gen Microbiol. 60:255.
192. Wells, A. G. (1937) Lancet 1:1221.
193. Whitehead, J. E. M., Wildy, P. and Engbaek, H. C. (1953) J. Pathol. and Bacteriol. 65:451.
194. Whitehead, J. E. M., Morrison, A. R. and Young, L. (19) Biochem. J. 51:585.
195. Wolinsky, E., Smith, M. M., Mitchell, R. S. and Steenken, W., Jr. (1957) Amer. Rev. Resp. Dis. 75:180.
196. Wolinsky, E., Smith, M. M. and Steenken, W., Jr. (1957) Amer. Rev. Tub. 76:497.
197. Wright, G. L., Affronti, L. F. and Reich, M. (19) Infect. Immun. 5:482.
198. Yanofsky, C. (1954) J. Bacteriol. 68:577.
199. Yegian, D. and Vanderlinde, R. J. (1947) J. Bacteriol. 54:577.
200. Young, L., Morrison, A. R. and Whitehead, J. E. M. (1952) Nature 169:711.
201. Young, W. D., Jr., Maslansky, A., Letar, M. S. and Kronish, D. P. (1970) Appl. Microbiol. 20:939.

CHAPTER 2

Ultrastructural and Chemical Features of the Mycobacterial Cell

Introduction

Electron microscopic studies indicate that the mycobacterial cells are prokaryotic. Thus, the nuclear material consists of deoxyribonucleic acid which is not separated from the cytoplasm by a membrane, and the respiratory processes in these cells are not associated with complex, membrane-bound intracellular structures, but are located in the protoplasmic membrane.

The most characteristic chemical feature of the mycobacteria is their high lipid content, which may amount to 20 to 40% of their dry weight (Table 1), and the presence of complex and unique lipids. The high lipid content of the tubercle bacilli was discovered very early (*4*); it attracted the attention of students of tuberculosis not only from the chemical point of view but also because of the possible association between this unique feature and the pathogenesis of tuberculosis. Investigators also realized that one of the most striking properties of the mycobacteria, their acid-fastness, might have something to do with their lipid constituents (*3,6,16,52*).

TABLE 1. Lipid Fractions from Acid Fast Bacilli
[adapted from Anderson, *5*]

	Organisms									
	Human[1]		**Avian[2]**		**Bovine[3]**		**Timothy[4]**		**Leprosy[5]**	
Lipids	**Gm**	**%**	**Gm**	**%**	**Gm**	**%**	**Gm**	**%**	**Gm**	**%**
Phosphatide	253.1	6.54	79.7	2.26	60.5	1.55	18.7	0.59	100.5	2.20
Acetone Soluble Fat	240.0	6.20	77.3	2.19	131.7	3.34	87.4	2.75	289.5	6.47
Chloroform Soluble Wax	427.0	11.03	379.5	10.79	336.0	8.52	158.4	4.98	444.8	9.98
Total Lipids	920.0	23.78	538.5	15.26	528.2	13.40	264.5	8.37	834.6	18.70
Dry Bacterial Residue	2902.0	75.01	2942.7	83.71	3370.1	85.50	2783.1	87.70	3389.8	80.38

[1] ***M. tuberculosis,*** strain H_{37}

[2] ***M. avium***

[3] ***M. bovis***

[4] ***M. phlei***

[5] Not ***M. leprae,*** but an acid fast organism isolated from a patient with leprosy.

The chemistry of the mycobacteria has been reviewed by many authors (*6,7,25,33,56*). These reviews are very extensive and complete, and therefore the subject need not be dealt with here. Instead, this chapter is mainly concerned with more recent information regarding the anatomy of the mycobacterial cell and the chemistry of the identified subcellular structures (cell wall, protoplasmic membrane, ribosome and nucleus).

The Cell Wall

The shape of the cell is maintained by a thick and rigid wall composed of two electron-dense layers separated by a less dense one (Figure 1). Removal of the cell-wall structures by various treatments converts the rod-shaped cells into spherical structures or spheroplasts (*71,73,96,97*), provided that the osmotic pressure in the surrounding medium is conveniently adjusted to equilibrate the internal pressure in the cell.

Electron microscopic studies reveal the presence of paired, fibrous structures on the surface of the cell envelope. These fibers measure about 50-80 Å[1] in width, and they appear to be characteristic of all the mycobacteria (*90,91*). Their function and chemical structure remain unresolved.

The cell wall consists of three distinct layers. The outer and inner layers are denser than the middle one. The thickness of the wall is about 100 to 200 Å. Chemical analysis of cell wall preparations reveals the presence of neutral sugars (arabinose and galactose, sometimes mannose), amino sugars (glucosamine and muramic acid, sometimes galactosamine), amino acids (D- and L-alanine, D-glutamic and *meso*-diamino pimelic acid), and mycolic acids (Table 2). The glucosamine-muramic acid molar ratio is about 1.0. The amino acid molar ratios found were 2 alanine, 1 *meso*-DAP, and, in most instances, 1 glutamic acid, but the amount of glutamic acid in two virulent strains of *M. tuberculosis* was found to be unusually high. The muramic acid in the cell walls of *M. tuberculosis, M. bovis,* BCG, *M. kansasii, M. smegmatis,* and *M. phlei* is glycolylated rather than acetylated as it usually is in other bacteria (*1,10,35,66,88*). Glycolylmuramic acid was also found in the Nocardia (*35*) but not in *Streptomyces albus* and *Cornyebacterium fermentans* (*10*).

Current concepts attribute the shape of the bacterial cells to the rigidity of the cell wall, which, in turn, is due to the architectural arrangement of a complex heteropolymer (murein, mucopeptide or peptidoglycan). The peptidoglycan was defined as "the insoluble material from isolated cell walls that under the electron microscope has the general outline of the cell from which it is isolated, and which can be shown to have a constant composition after repetition of suitable extraction procedures and to contain only amino sugars and a limited number of amino acids, which, when added together make up 100% (within experimental error of the methods used) of the weight of the material" (*70*). ~~So~~ far, in our laboratories, treatment of mycobacterial cell wall preparations with organic solvents, mild acid or alkali hydrolysis and various enzymes has failed to produce a structure fitting the above definition. Mycolic acids and reducing sugars remain consistently present in purified cell walls and represent the major portion of these wall preparations. However, the existence of a true peptidoglycan might be inferred from the following lines of evidence:

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[1] Å (Angstrom) = 10^{-10} meters

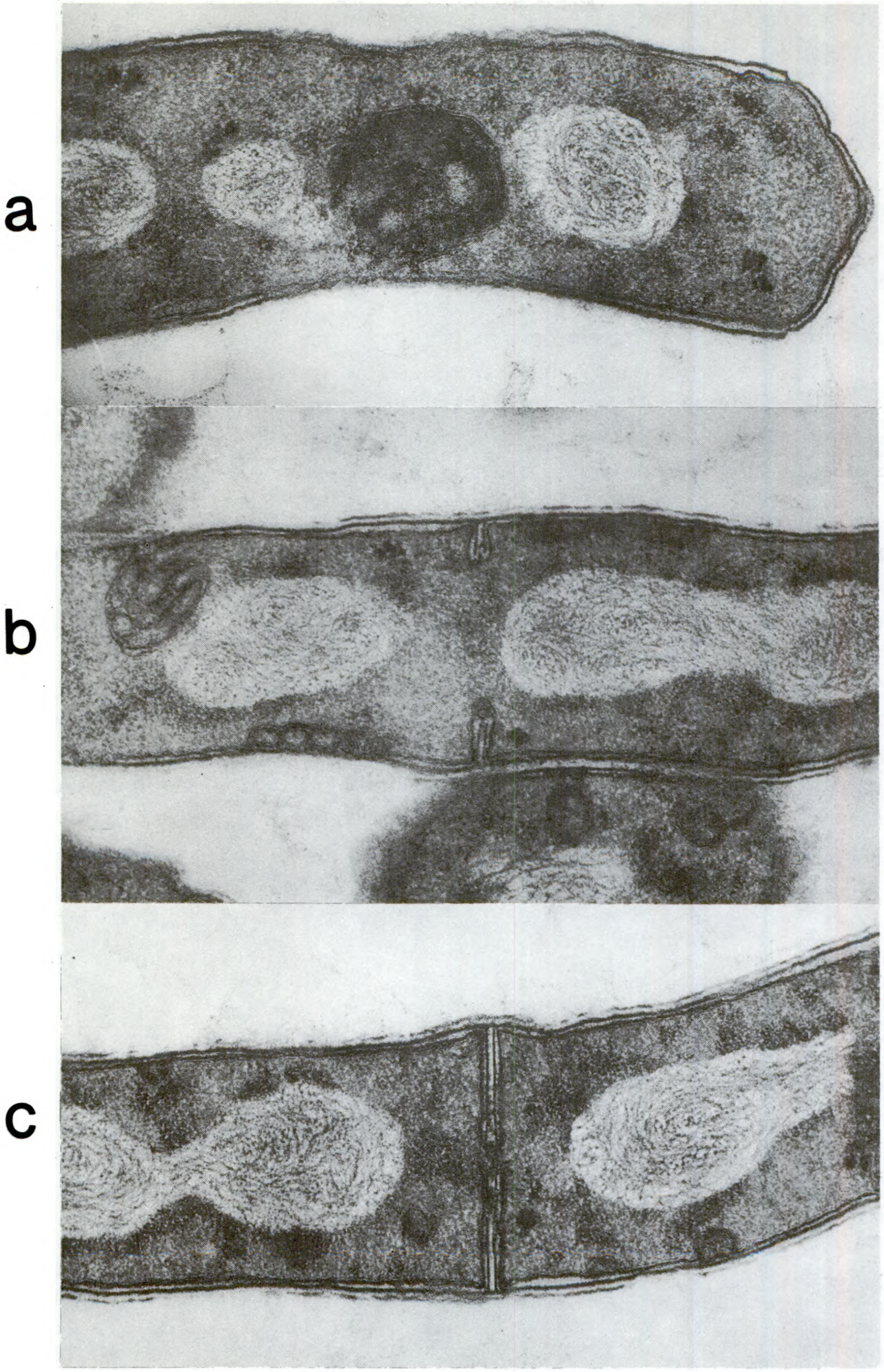

FIGURE 1. Ultra thin sections of *M. smegmatis* (dividing cells). Note the 3-layered cell wall and the well-defined underlying membrane, the nuclear regions and the mesosome. In A, note that the mesosome is continuous with the cell membrane. In B and C, note the cross wall (septum) as an inward invagination (courtesy of Dr. K. Takeya).

1. The amino sugar and amino acid composition and the respective molar ratios (Table 2) are consistent with a peptidoglycan, perhaps forming a rigid basal layer.

2. After partial solubilization of purified cell walls with the myxobacter AL_1 enzyme, an amidase that cleaves the bond between muramic acid and alanine, Petit *et al.* (*66*) obtained the tripeptide alanine-glutamic acid-DAP, the tetrapeptide alanine-glutamic acid-DAP-alanine, and a tetrapeptide alanine-glutamic acid-DAP-alanine, and a tetrasaccharide and a disaccharide, each of which gave equimolecular amounts of glucosamine and muramic acid.

3. Inhibitors of cell-wall biosynthesis induce the intracellular accumulation of putative peptidoglycan intermediates. Thus, D-cycloserine induces the accumulation of a UDP-N-glycolylmuramyl-tripeptide (*88*), as well as earlier precursors (*21*) in *M. tuberculosis* and in *M. phlei* (*67*), whereas glycine and its peptides were shown to induce the accumulation of UDP-acetylamino sugars in *M. smegmatis* (*106*).

How the mycolic acids and the reducing sugars are linked to the peptidoglycan to form a three-dimensional structure is still unclear. Kanetsuna (*45*) referred to the complete structure as a mycolic acid-arabino-galactan-mucopeptide complex. In this complex, the mycolic acids are esterified to the position 5 of arabinofuranose (*9,46*). Analysis of the arabino-galactan in *M. bovis* BCG indicated a branched structure consisting of 1→5 linked D-arabinose and 1→4 linked D-galactopyranose units, the side chain of which terminates by D-arabino-furanose residues (*62*). Similar arrangement was found in the *M. phlei* arabino-galactan (*63*). Muramic acid phosphate was identified in the cell wall of various mycobacteria, an observation that led to the suggestion of phosphodiester linkages between the peptidoglycan and the arabinogalactan (*45,66*). Proposed structures of the mycolic acid-arabino-galactan-mucopeptide complex are shown in Figure 2.

TABLE 2. Composition of Mycobacterial Peptidoglycans

		Amino Acids[2] Molar Ratios				
Spcies	**Gln / Mur[1]**	**Ala**	**Glu**	**DAP**	**Glyc**	**References**
M. lepraemurium		2.0	1.7	0.6	1.2	19
M. tuberculosis		2.0	1.6-20.0	0.9-1.4	0-0.5	12,61
M. bovis, BCG	1.0-1.1	2.0	1.1-2.0	0.9-1.3	0.35-1.0	45,50,51,61
M. kansasii	0.8	2.0	1.5	1.1	0.2	24
M. avium		2.0	1.3-1.4	0.68-1.23	0.72	12,61
M. fortuitum	0.9	2.0	2.0-2.2	0.9-1.1	0-0.6	12,24
M. phlei	1.2	2.0	1.4	1.1	0.2	92
M. smegmatis	1.2	2.0	1.0	0.9	0	45

[1] Glucosamine/muramic acid molar ratios

[2] Ala. = alanine; Glu = glutamic acid; DAP = *meso* diaminopimelic acid; GLYC = glycine.

The majority of the mycobacterial lipids seem to occur in the cell's outer layer (*50,51*). The relationship of one of these lipids, the Wax D, to the structure of the cell wall proper is of considerable interest. Most of the Wax D is extracted from the cells by organic solvents (loosely bound Wax D), but some remains as an integral part of the wall (bound Wax D). A procedure for lipid extraction from the mycobacteria is shown in Figure 3. The extracted Wax D is not a chemically homogeneous substance, but a mixture of related compounds sharing common solubility properties. Since they have different molecular weights, the various components could be separated by ultracentrifugation in ether (*44*) or by column chromatography in silicic acid of acetylated derivatives (*93*). All of the mycobacteria

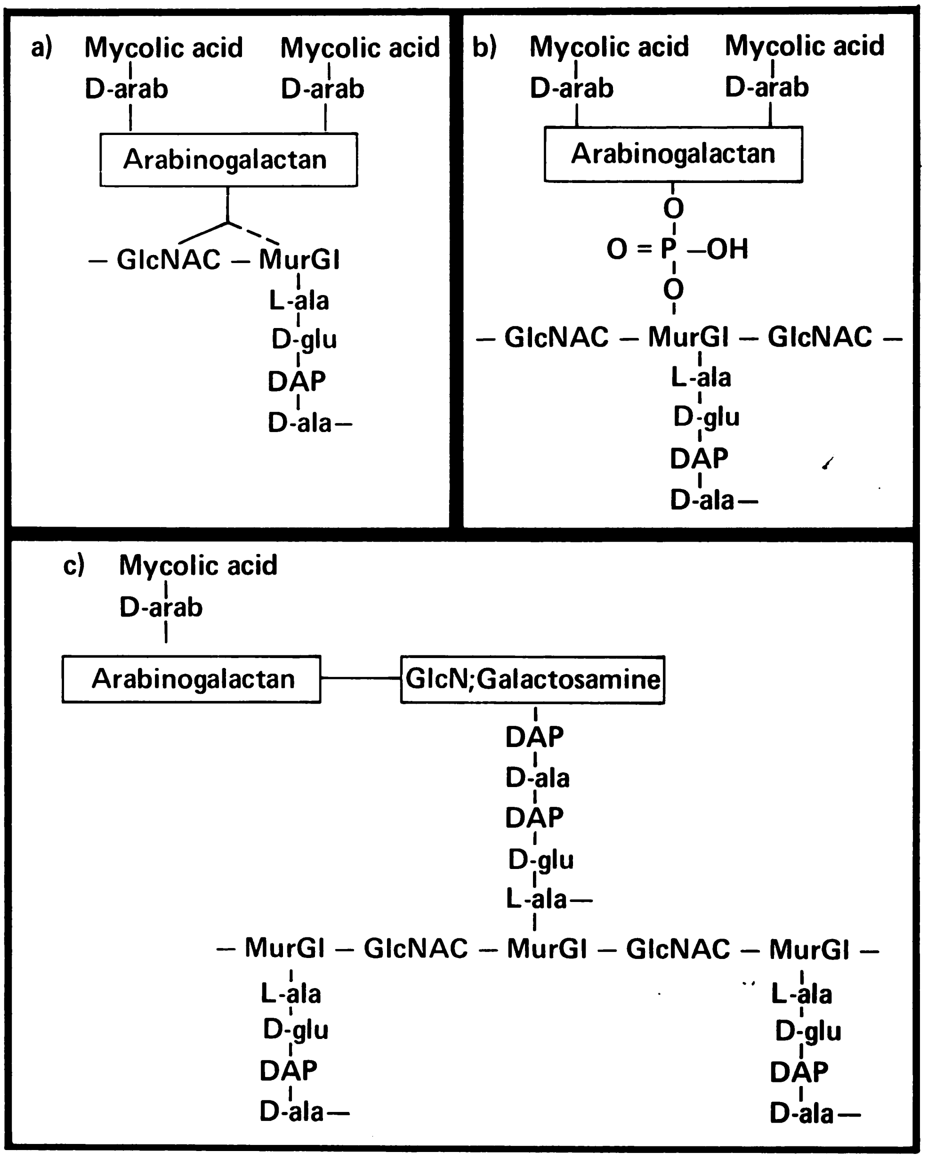

FIGURE 2. Proposed structures of the mycolic acid-arabino-galactan-mucopeptide complex of the mycobacterial cell wall. In a, the linkage between the arabinogalactan mycolate and the peptidoglycan is assumed to be a glycosidic linkage; in b, a phosphodiester linkage; in c, a peptide bridge. Structures a and b were proposed by Kanetsuna and San Blas (*47*). Structure c is based upon the isolation of GalactN-DAP by Jolles *et al.* (*44*), and the inhibition of Wax D synthesis by D-cycloserine (*23*).

contain Wax D, but only in *M. tuberculosis* and *M. kansasii* is a peptidoglycolipid a major component of the extractable Wax D; in other mycobacteria, the Wax D's are chiefly glycolipids (*53*).

The Wax D peptidoglycolipid of *M. tuberculosis* is a complex molecule with a molecular weight of about 140,000 (Figure 4). The structure of the peptidoglycolipid has a remarkable resemblance to the structure of the cell wall peptidoglycan. As Kotani *et al.* (*51*) suggested, the bound Wax D is an integral part of the cell wall, and it seems to correspond to Kanetsuna's mycolic acid-arabinogalactan-mucopeptide complex described above. Further evidence of this intimate relationship is provided by the finding that D-cycloserine blocks the biosynthesis of both heteropolymers in *M. tuberculosis* (*21,23*).

We have indicated above that most lipids appear to be located at the cell's outer layers. There are two other classes of lipids (the mycosides and the "cord factor") whose presence is known to alter the cell surface in distinct ways.

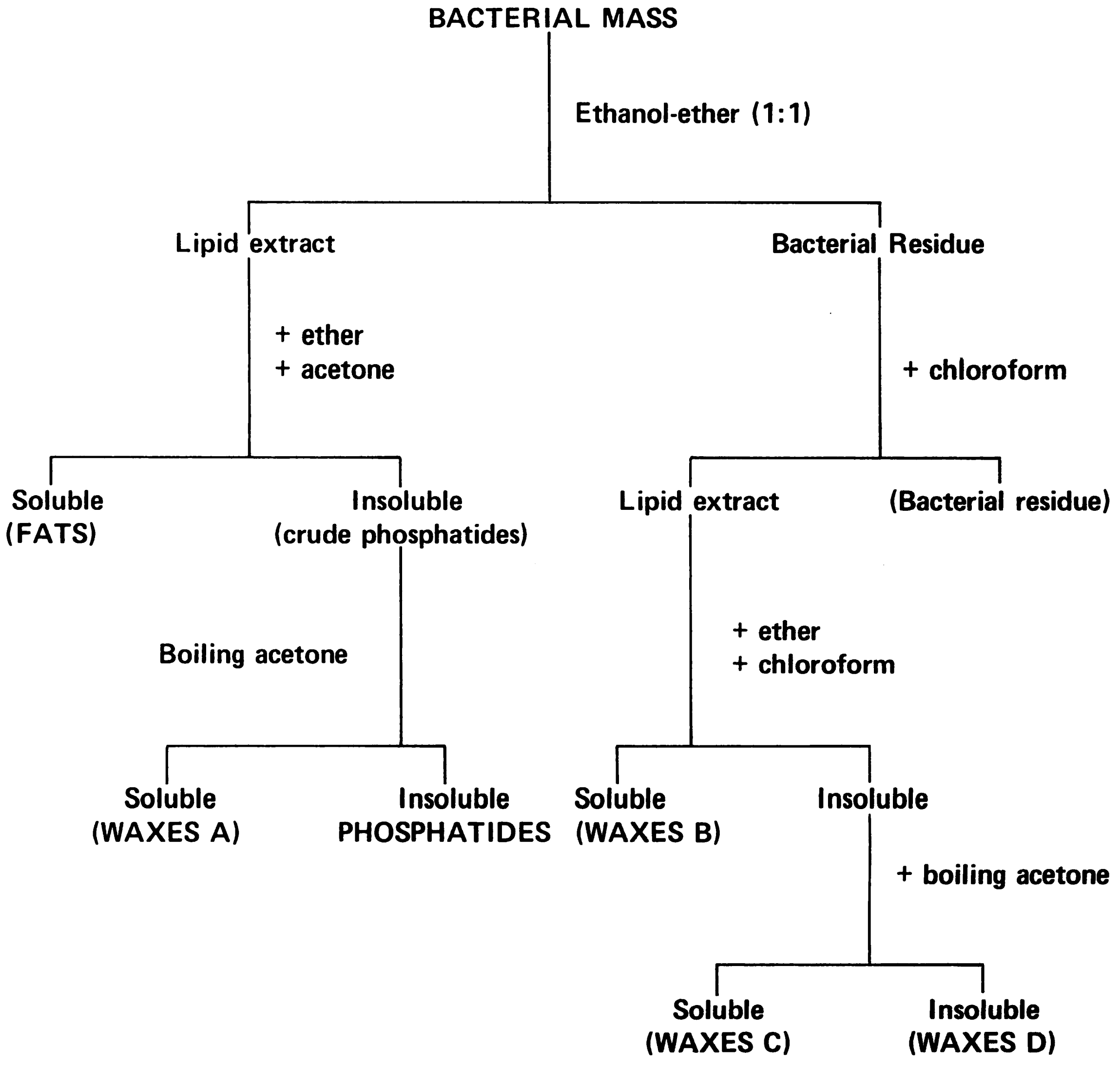

FIGURE 3. Procedure for the extraction of mycobacterial lipids, according to Anderson (adapted from Asselineau, *7, 8*).

The mycosides represent a group of complex lipids that were defined as species specific glycolipids or peptidoglycolipids of mycobacterial origin (*82*). These lipids appear to be located at the cell's surface, since they are extracted in larger amounts from cells that yield smooth colonies on solid media (*83,84*). They are characterized by their infrared spectrum and were found in *M. bovis* (mycoside B), *M. kansasii* (mycoside A), *M. fortuitum* (mycoside F), *M. paratuberculosis* (mycoside J_2), *M. avium* (mycoside C_2), *M. scrofulaceum* (mycoside Cm), *M. marinum* (mycoside G), *M. butyricum* (mycoside Cb), and an unidentified strain of a scotochromogenic mycobacterium (mycoside D). *M. tuberculosis* does not contain a mycoside. The biological role of these lipids is unknown.

The cells of virulent strains of *M. tuberculosis* tend to lie parallel to each other, forming tight cords. Koch (*49*) observed cording by *M. tuberculosis* in 1884, but only in 1947 was it shown that the formation of cords was characteristic of virulent strains of *M. tuberculosis* (*61*). Bloch (*13*) demonstrated that the extraction of these strains with petroleum ether yields a lipid that is toxic for mice, which he called "cord factor." Chemical studies on the structure of the "cord factor" showed it to be a $6,6^1$-2-D-trehalose mycolate. The possible biological significance of the "cord factor" is discussed later on in conjunction with the genetic changes associated with resistance to isoniazid in the tubercle bacilli.

By using knowledge gained from various chemical extractions and electron microscopy, Imaeda *et al.* (*40*) proposed a tridimensional model of the cell wall which is in good agreement with the observations of most students.

The Protoplasmic Membrane

Underneath the cell wall, a distinct unitary membrane can be seen in cell sections (Figure 1). The 30 Å membrane consists of two layers, separated by a low density space that is 3 Å wide. In dividing cells, the membrane forms an invagination which progresses inward until division is completed. Between the two layers of the invaginated membrane, there is the accumulation of a moderately dense substance that may represent the precursors of the cell wall. The membranous structure undergoes additional invagination and foldings to form a multilaminar structure resembling a mesosome. The unitary membrane appears as a distinct structure in plasmolyzed cells and in spheroplasts.

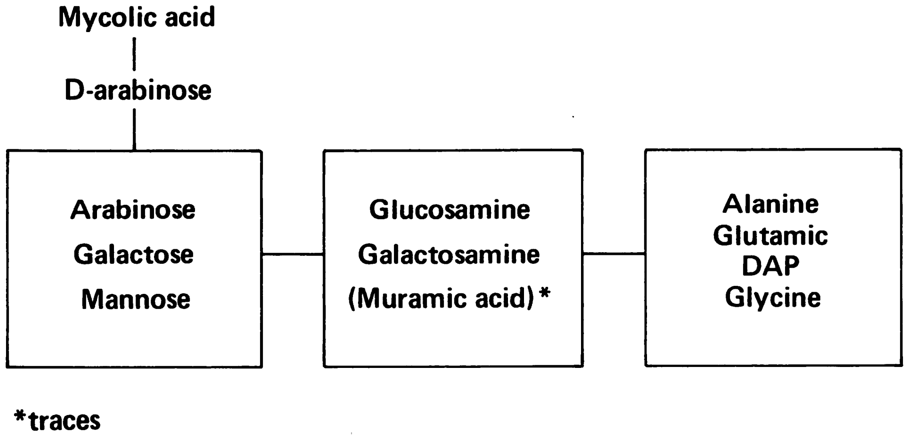

FIGURE 4. Structure of *M. tuberculosis* Wax D (modified from Jollès *et al.*, *44*).

The orderly sequence of events that occur during cell division was found to be disorganized in cells exposed to D-cyloserine (an inhibitor of the biosynthesis of the cell wall and the Wax D peptidoglycolipid in the tubercle bacilli), which adds further evidence to substantiate the role played by the membrane in the synthesis of the cell wall (*41*).

Membrane preparations are obtained by differential centrifugation of mechanically disrupted cells. These membrane preparations contain a large number of protein enzymes. Thus, almost all the respiratory activity, the cytochromes, NADH-oxidase, phospholipase A, and the enzymes catalyzing the biosynthesis of the mannophospholipids are found in these particulate preparations. These protein enzymes are discussed elsewhere in this book.

Chemically, the mannophospholipids (phosphoinositides, phosphatidyl-*myo*-inositol oligomannosides) are the most characteristic lipids in these membranes. Although these lipids are discussed here in relation to the cell's membrane, it should be noted that they are also present in the cell wall.

Studies of the mycobacterial phosphatides lead to the identification of cardiolipin, the phosphatidyl-*myo*-inositol oligomannosides phosphatidyl-ethanol amine and phosphatidic acid in *M. tuberculosis* (*2,29,86,87,99,101*). Cardiolipin was found in *M. phlei* and *M. avium* (*2,29*), the phosphatidyl-*myo*-inositol oligomannosides in *M. fortuitum, M. scrofulaceum,* and *M. phlei* (*2,59,100,102*), and phosphatidyl ethanolamine in *M. phlei* (*86*). Lee and Ballou (*54,55*) elucidated the chemical structure of these complex lipids. Of interest is the fact that these lipids were found only in the mycobacteria, in *Corynebacterium diphtheriae* and in *Nocardia braziliensis* (*38*).

The characteristic color of the cell mass of several of the mycobacteria is caused by the accumulation of carotenoid pigments. Like in other bacteria (*16a*) the carotenoids appear to be located on the cell membrane in the mycobacteria (*58*). The carotenoids usually found can be conceived to be composed of eight isoprene units (isoprenoid lipids containing 40 carbon atoms). When partitioned between petroleum ether and methanol, the less polar ones separate into the petroleum ether phase (epiphase) and the more polar ones separate into the methanol phase (hypophase). Generally, the epiphasic pigments are called carotenes, and the hypophasic are called xanthophylls. Among the mycobacteria, several carotenes and xanthopylls were found (Table 3). In addition to the species shown in Table 3, extracts from scotochromogenic mycobacteria analyzed by thin layer chromatography showed the occurrence of several pigments (*95*). However, the pigments were not characterized.

The biogenesis of the carotenoids in the mycobacteria is discussed in Chapter 3.

TABLE 3. Carotenoid Pigments of the Mycobacteria

Species	Carotenoids Identified	References
M. tuberculosis	Leprotene	89
M. marinum	β-carotene, lycopene, phytoene, phytofluene, α-carotene, ε-carotene, neurosporene	37,57
M. kansasii	β-carotene, δ-carotene, phytofluene, α-carotene, leprotene, lycopene, phytoene, neurosporene, ε-carotene, γ-carotene	20
M. phlei	Myxoxantophyll, lyopene, β-carotene, γ-carotene, phytoene, phytofluene, neurosporene, leprotene, zeaxanthin	75

The Ribosomes

Ultrastructural studies of cells at various stages of growth reveal the presence, in the cytoplasm, of small particles of about 80 x 120 Å whose number increases from the early logarithmic phase to the later stages of growth. These particles (ribosomes) are interconnected by fibrillae, measuring approximately 7 Å in diameter, to form polyribosomes (*42*). Chemical analysis has shown that the ribonucleic acid content of the cells also increases from the early stages to the late stages of growth (*109*), whereas the ribosome content at different stages of growth is also in agreement with that noted in the ultrastructural studies (*105*).

Differential centrifugation of mechanically disrupted cells showed that the ribosomes sediment along with the membrane fragments. Pure ribosome preparations were obtained by gradient centrifugation on a 5-20% sucrose gradient by Worcel *et al.* (*105*). These investigators showed that the mycobacterial ribosomes (*M. tuberculosis,* strain H_{37} Ra) were large 70 S particles that were dissociated by reducing the Mg++ concentration into smaller 50 S and 30 S particles.

Mycobacterial ribosomes were found to catalyse a poly U-directed incorporation of ^{14}C-phenyl alanine into protein (*98*).

The Nucleus

The nucleus of the mycobacteria consists of filaments which probably are a single, long, coiled DNA molecule, 30 Å across. Investigations of the mycobacteria DNA indicated it to be a double-stranded molecule with a very high guanine plus cytosine content (Table 4). The molecular weight of the mycobacterial DNA's (Table 5) was estimated to be 2.5 - 4.5 x 10^9 Daltons.

It appears from bacterial sections published in the literature that the mycobacteria are mononucleate organisms.

TABLE 4. Guanine + Cytosine (G + C %) Contents of the Mycobacterial DNA's [*94, 103, 104*]

Species	G + C %
M. avium	68.2
M. bovis, BCG	64.6
M. borstelense	61.6
M. diernhoferi	66.0
M. fortuitum	64.0
M. gordonae	65.8
M. intracellulare	63.0
M. kansasii	60.8
M. marinum	62.8
M. phlei	65.8
M. scrofulaceum	63.4
M. smegmatis	61.8
M. tuberculosis	65.0; 65.2

TABLE 5. Molecular Weight of Mycobacterial DNA's
[adapted from Bradley, *14*]

Source of DNA	Genome Size (x 10^9 Daltons)
M. avium	2.9
M. bovis, BCG	2.8
M. fortuitum	2.5
M. intracellulare	2.5
M. kansasii	3.8
M. marinum	3.9
M. phlei	3.4
M. smegmatis	4.2 - 4.5
M. tuberculosis, $H_{37}Ra$	2.5

Cell Division

Under usual laboratory conditions, mycobacteria multiply by binary division. A generalized view is that division is initiated when all critical macromolecules are present. As these critical components are synthesized and accumulate, the cell undergoes elongation, and division begins when the ratio of cell surface to cell volume reaches a critical stage (*77*). Studies undertaken with the electron microscope (*27,39,42,68*) show intrastructural changes in dividing cells that may be reconstituted into a sequence of events that progress in a regular and orderly fashion. During division, an intracytoplasmic membrane system is formed, apparently as the result of infoldings of the cytoplasmic membrane. The initial infoldings undergo additional invaginations, and multivescicular or lamellar structures, or mesosomes (*30*), are then formed (Figure 1). As the infoldings progress, newly synthesized cell wall material accumulates and a septum is formed. The annular ingrowth of the membrane and cell wall then proceeds until division is completed.

The above reconstruction of events that take place in cell division suggests that the synthesis of the cell wall material is localized, and that, therefore, each daughter cell contains cell wall from its parent as well as newly formed cell-wall material. Although this has been shown to be the case in *Streptococcus pyogenes* (*17*) and other bacteria, it appears that the gram-negative bacilli wall is formed by diffuse intercalation of newly synthesized wall material. Whether the wall in the mycobacteria grows by addition of material strictly localized at the division site or whether the deposition of the material occurs uniformly throughout the whole surface of the cell is not known.

The addition of new material to the closed tridimensional wall envelope and the separation of the daughter cells must require the existence of hydrolytic enzymes to split some of the covalent bonds. These enzymes are known as autolytic enzymes. Various "autolysins" have been described, which exhibit distinct specificities (*28,31,34,76,78,79,80, 81,107,108*). The site of action of the autolysins was suggested to be along the leading edge and site of the growing cell wall (*36*). Many bacteria are known to autolyse when growth ceases, whether the cessation of growth is caused by the exhaustion of a nutrient, by unbalanced growth conditions (*78*), or by agents like the penicillins that inhibit the synthesis of the cell wall but do not inhibit the autolytic enzymes (*76,77*). The mycobacteria are known to autolyze (*11,18,74,104*), but neither the exact conditions nor the mechanism of autolysis has been investigated in detail. Fragments of a cell wall peptidoglycan are released from *M. smegmatis* cell-wall preparations suspended in a buffer. The release of these fragments was enzymatic; however, the specificity of the enzyme(s) was not established (*48*).

The antibiotic D-cycloserine, a known inhbitor of cell-wall synthesis in the bacteria (*65,85*), induces the lysis of sensitive mycobacterial cells. In *M. tuberculosis* the drug was shown to inhibit the enzymes D-alanine and D-alanyl-D-alanine synthetase (*21,22*). Thus the synthesis of the cell wall peptidoglycan and the "Wax D" peptidoglycolipid (*21,23*), was interrupted and the intracellular accumulation of the putative intermediate precursor, UDP-N-*glycolyl* muramyl-D-glu-*meso* DAP-L-ala was induced (*67,88*). The final step in the inhibition of the wall synthesis is cell lysis. Studies with the electron microscope of cells exposed to D-cycloserine revealed the inward detachment of the plasma membrane and then protoplast formation and lysis (*41*). Whether bacteriolysis in this case is caused by autolytic enzymes remains to be seen. It should be emphasized that in the mycobacteria the solubilization of the peptidoglycan does not seem localized to the tip of the septum but seems to be diffuse along the innermost layer.

As cell division progresses, some mechanism must exist to assure that each daughter cell receives an exact replica of the mother cell chromosome. In other words, the replication of the DNA must proceed concurrently with cell division. A current hypothesis (*43*) is that the bacterial chromosome is attached to the membrane and the synthesis of the membrane at the point of attachment brings about the separation of the two daughter chromosomes. Thus, the membrane would function as a rudimentary mitotic apparatus. This subject was recently reviewed by Ryter (*72*).

In spite of its importance, the study of cell division and its control in the mycobacteria has not received much attention.

REFERENCES

1. Adam, A., Petit, J. F., Wietzerbin-Falszpan, J., Sinay, P., Thomas, D. W. and Lederer, E. (1969) FEBS Letters 4:87.
2. Akamatsu, Y., Ono, Y. and Nojima, S. (1965) J. Biochem. 61:96.
3. Anderson, R. J. (1929) J. Biol. Chem. 85:505.
4. Anderson, R. J. (1932) Physiol. Rev. 12:166.
5. Anderson, R. J. (1943) Yale J. Biol. and Med. 15:311.
6. Asselineau, J. (1951) Thesis, Paris, Ed. Librairie Arnette, Paris.
7. Asselineau, J. (1952) Adv. Tub. Res. 5:1.
8. Asselineau, J. (1966) The Bacterial Lipids, Holden-Day Inc., Publishers, S. Francisco, California, 1966.
9. Azuma, I., Tamamura, Y. and Misuki, A. (1969) J. Bacteriol. 98:331.
10. Azuma, I., Thomas, D. W., Adam, A., Ghuysen, J. M., Bonaly, R., Petit, J. F., and Lederer, E. (1970) Biochem. Biophys. Acta 208:444
11. Baisden, L. and Yegian, D. (1943) J. Bacteriol. 45:163.
12. Belknap, D., Camien, M. and Dunn, M. (1961) Proc. Soc. Exptl. Biol. and Med. 107:289.
13. Bloch, H. (1950) J. Exptl. Med. 91:97.
14. Bradley, S. G. (1972) Amer. Rev. Resp. Dis. 166:122.
15. Brieger, E. M. and Fell, H. D. (1952) J. Gen. Microbiol. 7:287.
16. Choucroun, N. (1948) Compt. Rend. Sc. Soc. Biol. 226:1477.

16a. Ciegler, A. (1965) Appl. Microbiol. 7:1.

17. Cole, B. M. and Hahn, J. J. (1962) Science 135:722.
18. Corper, H. J. and Sweany, H. C. (1918) J. Bacteriol. 3:129.
19. Cummins, C. S. and Atfield, G. (1967) J. Gen. Microbiol. 49:377.
20. David, H. L. (1974) Appl. Microbiol. 28:696.
21. David, H. L. (1969) Ph.D. Thesis, The University of Wisconsin, Madison, Wisconsin.
22. David, H. L., Takayama, K. and Goldman, D. S. (1969) Amer. Rev. Resp. Dis. 100:579.
23. David, H. L., Goldman, D. S. and Takayama, K. (1970) Infect. and Imm. 1:74.
24. De Wijs, H. and Jolles, P. (1964) Biochem. Bioph. Acta 83:326.
25. Drea, W. F. and Andrejew, A. (1953) The metabolism of the tubercle bacillus, Charles C. Thomas, Publishers, Springfield, Ill., 1953.
26. Ebina, T., Motomiya, M. Munakata, K. and Satake, O. (1962) Amer. Rev. Resp. Dis. 86:740.
27. Edwards, R. P. (1970) J. Med. Microbiol. 3:493.
28. Fan, D. (1970) J. Bacteriol. 103:494.
29. Faure, M. and Marechal, J. (1962) Compt. Rend. Sc. Soc. Biol. 254:4518.
30. Fitz-James, P. C. (1960) J. Biophys. Biochem. Cytol. 8:507.
31. Forsberg, C. and Rogers, H. J. (1971) Nature 229:272.
32. Goodwin, T. W. and Jamikorn, J. (1956) Biochem. J. 62:269.
33. Goren, M. B. (1972) Bacteriol. Rev. 36:33.
34. Goyette, J. and Ghuysen, J. M. (1970) Biochem. 9:2952.
35. Guinand, M., Vacheron, M. J. and Michel, G. (1970) FEBS Letters 6:37.
36. Higgins, M. L., Pooley, H M. and Shockman, G. D. (1970) J. Bacteriol. 103:504.
37. Howes, C. D. and Batra, P. P. (1970) Biochem. Biophys. Acta 222:174.
38. Ikawa, M. (1967) Bacteriol. Rev. 31:54.
39. Imaeda, T. and Ogura, M. (1963) J. Bacteriol. 85:150.
40. Imaeda, T., Kanetsuna, F. and Galindo, B. (1968) J. Ultrast. Res. 25:46.
41. Imaeda, T., Kanetsuna, F. and Rieber, M. (1968) Tubercle 49:385.
42. Imaeda, T., Kanetsuna, F., Rieber, M., Galindo, B. and Cesari, I. M. (1969) J. Med. Microbiol. 2:181.
43. Jacob, F., Brenner, S. and Cuzin, F. (1963) Cold Spring Harb. Symp. Biol. 28:329.
44. Jolles, P., Samour, D. and Lederer, E. (1962) Arch. Biochem. Biophys. Sup. 1, p. 283-289.
45. Kanetsuna, F. (1968) Biochem. Biophys. Acta 158:130.
46. Kanetsuna, F., Imaeda, T. and Canto, G. (1969) Biochem. Biophys. Acta 173:341.
47. Kanetsuna, F. and San Blas, G. (1970) Biochem. Biophys. Acta 208:434.
48. Kilburn, J. O. (1974) Ph.D. Thesis, Medical College of Georgia, Augusta, Georgia.
49. Koch, R. (1884) Mitt. K. Gesundheit 2:1.
50. Kotani, S., Kitaura, T., Hirano, T. and Tanaka, A. (1959) Biken J. 2:129.

51. Kotani, S., Hashimoto, S., Matsubara, T., Kato, K., Harada, K., Kogami, J., Kitaura, T. and Tanaka, A. (1963) Biken J. 6:181.
52. Lammana, C. (1946) J. Bacteriol. 52:99.
53. Lederer, E. (1967) Chem. and Phys. Lipids 1:294.
54. Lee, Y. C. and Ballou, C. E. (1964) J. Biol. Chem. 239:1316.
55. Lee, Y. C. and Ballou, C. E. (1965) Biochem. 4:1395.
56. Long, E. (1958) The Chemistry and Chemotherapy of Tuberculosis, The Williams and Wilkins Company, Baltimore, Md.
57. Matthews, M. M. (1963) Photochem. and Photobiol. 2:1.
58. McCarthy, C. (1971) Appl. Microbiol. 22:546.
59. Michele, G. and Lederer, E. (1955) Compt. Rend. Sc. Soc. Biol. 240:2454.
60. Middlebrook, G., Dubos, R. J. and Pierce, C. (1947) J. Exptl. Med. 76:175.
61. Migliore, D., Acharya, N. and Jolles, P. (1966) Compt. Rend. Sc. Acad. Sc. 263:846.
62. Misaki, A. and Ikawa, N. (1966) J. Biochem. (Tokyo) 59:511.
63. Misaki, A., Ikawa, N., Kato, T. and Kotani, S. (1970) Biochem. Biophys. Acta 215:405.
64. Noll, H., Bloch, H., Asselineau, J. and Lederer, E. (1956) Biochem. Biophys. Acta 20:299.
65. Park, J. T. (1958) Biochem. J. 70:2 P.
66. Petit, J. F., Adam, A., Wietzerbin-Falszpan, J., Lederer, E. and Ghuysen, J. M. (1969) Biochem. Biophys. Res. Comm. 35:478.
67. Petit, J. F., Adam, A., and Wietzerbin-Falszpan, J. (1970) FEBS Letters 6:55.
68. Petitprez, A., Roos, P. and Tacquet, A. (1967) J. Microscopy 6:229.
69. Previc, E. P. (1970) J. Theoret. Biol. 27:471.
70. Rogers, H. J. and Perkins, H. R. (1968) "Cell Walls and Membranes" E & F. N. Spon. Ltd., London, 1968, p 240.
71. Roos, P., Gaudier, B., Tacquet, A. and Guillaume, J. (1966) Ann. Inst. Pasteur (Lille) 30:49.
72. Ryter, A. (1968) Bact. Rev. 32:39.
73. Sato, H., Kiena, B. B. and Greenberg, L. (1965) Canadian J. Microbiol. 11:807.
74. Schaeffer, W. B., Marshak, A. and Burkhart, B. (1949) J. Bacteriol. 58:549.
75. Schlegel, H. G. (1959) J. Bacteriol. 77:310.
76. Schwarz, V. and Weidel, W. (1965) Z. Naturf. 206:147,153.
77. Schwarz, V., Asmus, A. and Frank, H. (1969) J. Mol. Biol. 41:419.
78. Shockman, G. D. (1965) Bacteriol. Rev. 29:345.
79. Shockman, G. D., Pooley, H. M., and Thompson, J. S. (1967) J. Bacteriol. 94:1525.
80. Shockman, G. D., Thompson, J. S. and Conover, M. J. (1967) Biochem. 6:1054.
81. Shockman, G. D. and Cheney, M. C. (1969) J. Bacteriol. 98:1199.
82. Smith, D., Randall, H., MacLennan, A. and Lederer, E. (1960) Nature 186:887.
83. Smith, D., Harrell, W. and Randall, H. (1954) Amer. Rev. Tub. 69:505.
84. Randall, H. and Smith, D. (1964) Zentralbl. Bakteriol. 194:190.
85. Strominger, J., Threnn, R. and Scott, S. (1959) J. Am. Chem. Soc. 81:3803.
86. Subrahmayan, D., Nandedkar, A. H. and Viswanathan, R. (1962) Biochem. Biophys. Acta 63:542.
87. Subrahmayan, D. (1964) Canad. J. Biochem. 42:1195.
88. Takayama, K., David, H. L., Wang, L., and Goldman, D. S. (1970) Biochem. Biophys. Res. Comm. 39:7.
89. Takeda, Y. and Ohta, T. (1951) Chem. Abstr. 45:1198.
90. Takeya, K., Mori, R., Koike, M. and Toda, T. (1958) Biochem. Biophys. Acta 30:197.
91. Takeya, K., Mori, R., Tokunaga, T., Koike, M. and Hisatsune, K. (1961) J. Biophy. Biochem. Cytol. 9:496.
92. Takeya, K., Hisatsune, K. and Inove, Y. (1963) J. Bacteriol. 85:24.
93. Tanaka, A. (1963) Biochem. Biophys. Acta 70:483.
94. Tarnok, J., Röhrscheidt, E., and Bönecke, R. (1967) Rassegna di Pathologia dee Apparato Resp. 17:4.
95. Tarnok, I. and Tarnok, Zs (1971) Tubercle 52:127.
96. Thacore, H. and Willet, H. P. (1963) Proc. Soc. Exp. Biol. Med. 114:43.
97. Thacore, H. and Willet, H. P. (1966) Amer. Rev. Resp. Dis. 93:786.
98. Trnka, L., Wiegeshaus, E. and Smith, D. W. (1968) J. Bacteriol. 95:310.
99. Vilkas, E. (1957) Compt. Rend. S. Soc. Biol. 245:588.

100. Vilkas, E. and Lederer, E. (1955) Compt. Rend. S. Soc. Biol. 240:1156.
101. Vilkas, E. and Lederer, E. (1956) Bull. Soc. Chem. Biol. 38:111.
102. Vilkas, E. and Rojas, A. (1964) Bull. Soc. Chem. Biol. 46:689.
103. Vischer, E., Zamenhof, S. and Chargaff, E. (1949) J. Biol. Chem. 177:429.
104. Wayne, L. and Gross, W. M. (1968) J. Bacteriol. 95:1481.
105. Worcel, A., Goldman, D. S. and Sacks, J. B. (1968) Proc. Nat. Acad. Sc. 61:122.
106. Yabu, K. (1969) Biochem. Biophys. Acta 184:460.
107. Young, F. E. (1963) J. Biol. Chem. 238:319.
108. Young, F. E. (1966) J. Bacteriol. 92:839.
109. Youmans, A. S. and Youmans, G. P. (1968) J. Bacteriol. 95:272.

CHAPTER 3

Metabolism of the Mycobacteria

Introduction

Most mycobacteria will grow in simple media. Thus, the tubercle bacilli can grow in a medium composed of glucose, an ammonium salt, magnesium sulfate and potassium phosphate (*57,58*). The mycobacteria that can be cultivated in the laboratory do not require vitamins, other co-factors or complex growth factors, with the noticeable exception of *M. paratuberculosis* which requires mycobactin (*73*). These organisms are prototrophic for all the amino acids and purine and pyrimidine bases. The energy that is required for their growth is obtained by the oxidation of an organic substrate (either glucose or glycerol), and they depend on oxidation-reduction reactions to generate energy. Only a few elements (potassium, sulphur, phosphorus and magnesium) are necessary.

Although the mycobacteria are chemoorganotrophic and depend only on glucose, or preferably glycerol, as the oxidizable substrate, the yield in cell mass can be greatly increased by enriching the medium with protein hydrolysates and some oligoelements (iron, copper, zinc and manganesium). Examples of chemically defined media are shown in Table 1.

Comprehensive reviews on the nutritional requirements of the mycobacteria and on the necessary growth conditions (pH, temperature, and concentration of oxygen and carbon dioxide) have been written by Drea and Andrejew (*21*), Long (*50*), and Darzins (*15*). This chapter deals with the biochemical aspects of mycobacterial growth rather than with the nutrition of these bacteria. The books mentioned above contain a considerable amount of information on nutrition. Edson (*23*), Goldman (*26*) and Ramakrishnam *et al.* (*66a*), have reviewed the intermediary metabolism of the mycobacteria.

TABLE 1. Examples of Chemically Defined Media Used in Mycobacteriology

Compound (grams)	Long (50)	Merril (57)	Sauton (15)	Proskauer & Beck (66)	Goldman (26)
Ammonium sulfate		5.0			
Asparagine	5.0		4.0	5.0	
Ammonium citrate	5.0				
Ferric ammonium citrate	0.005		0.05	0.05	0.1
Ammonium chloride					4.0
Casitone					0.6
Glycerol	50.0	5.0	35.0	15.0	40.0
Citric acid			2.0		4.0
Potassium sulfate					1.2
Potassium phosphate (acid)	3.0	0.4		5.0	
Dipotassium phosphate			0.5		8.0
Disodium phosphate		1.6			
Sodium carbonate (anhydrous)	3.0				
Sodium glutamate					3.5
Sodium chloride	2.0	5.0			
Magnesium sulfate	1.0	0.05	0.5	0.6	
Magnesium citrate				2.5	
Magnesium chloride					2.4
Tween 80 (ml)					2.0 of 20% solution
Water to (ml)	1,000	1,000	1,000	1,000	2,000
pH	7.0	7.0	7.4	7.0	6.6

Growth Curve

The growth curve of the mycobacteria in liquid media can be divided into six phases: the lag, acceleration, exponential, arithmetic linear, negative acceleration and stationary phases of growth (*24*). During the exponential phase, the cell mass and every other chemical constituent of a culture increase by a constant factor, and the culture is said to be in *balanced growth.*

The increase in cell mass at a constant rate is expressed mathematically by equation 1:

$$dB/dt = kB$$

in which B is the cell mass, t is time, and k is the growth rate constant.

The cell mass B may be expressed in various ways (cell number, cell weight, total nitrogen, or some other convenient parameter). Usually, B is expressed as the viable cell number N (see Chapter 4), and the above equation is converted by integration to equation 2:

$$N_t = No \bullet e^{kt}$$

in which N_t is the viable count at time t, No is the viable count at time zero, e is the base of the natural logarithm, and k has the same meaning as above. By taking the logarithm, equation 3 is obtained:

$$k \bullet t = \ln \frac{Nt}{No} \text{ or } k \bullet t = 2 \bullet 3 \log_{10} \frac{Nt}{No} \text{ (for } \ln A = 2 \bullet 3 \log_{10} A\text{).}$$

The doubling time (mean generation time, G), or the time the populations increase from No to 2 • No, is obtained by substituting 2 for $\frac{Nt}{No}$ which yields $G = \frac{0.69}{k}$.

The above equations are applicable at the exponential phase of growth which occurs only if the cells are maintained well dispersed. Since the mycobacteria tend to clump, Youmans and Youmans (*92*) proposed a method for determining the growth rate whose design circumvents this complication. The method consists of inoculating a series of tubes containing a liquid medium with graded amounts of cells (wet weights). The cultures are then incubated and the time it takes for growth to be visible is recorded. The slope obtained when the time is plotted against the weight of the inocula represents the growth rate constant (k). The generation time G is given: $G = \frac{0.69}{k}$.

The doubling times in the mycobacteria (Table 2) spread from about 2.3 hours in *M. phlei* to several days in *M. leprae* (determined by the mouse footpad method) (*18a,72*).

The exponential phase of growth is followed by a period of constant decrease in growth rate, a plot of which exhibits a shape described by Fisher *et al.* (*24*) as the arithmetic linear phase of growth. This is characteristic of all the mycobacteria, and suggests that a growth limiting factor becomes established in the cultures. Various investigators (*23,24,26,92*) hypothesized that the growth limiting factor was an inadequate diffusion of oxygen in the medium, which could be minimized by growing the cells under conditions of forced aeration (shaking or rotary cultures, or forced bubbling of air). However, the beneficial effect of aeration appears to depend on the nature of the oxidizable substrate. Thus, aeration of cultures of tubercle bacilli in medium containing glucose was found to cause inhibition of growth, but in medium containing glycerol, the exponential phase of growth was extended (*51,81*). To date, the fundamental aspects of this characteristic phase of growth have not been satisfactorily elucidated.

During the stationary phase of growth, probably initiated by the depletion of nutrients in the medium, autolysis does occur. It becomes apparent and occurs almost constantly in cultures in media made deficient in one nutrient. For example, in medium in which nitrogen is made growth limiting by reducing the concentration of the nitrogen source, autolysis begins shortly after the population reaches the stationary phase (*33*).

TABLE 2. Generation Times (G) of Some Mycobacteria

Species	**Medium**	**(G, in hrs^{-1})**	**References**
M. tuberculosis	Mice	20.4 - 21.9	68
	7H9	15.0	18a
	Lowenstein-Jensen	18.06	32
	Lowenstein	19.27-20.47	32
	Petragnani	24.07	32
M. fortuitum	7H9	4.6	18a
M. phlei	7H9	2.3	18a
M. marinum	7H9	8.0	18a
M. kansasii	7H9	13.0	18a
M. smegmatis	7H9	3.2	18a
M. flavescens	7H9	7.6	18a
M. leprae	Mouse foot pad	12.5 days	72

The existence of two phases of growth in which there is an increase in cell mass is of considerable importance in interpreting biochemical and other data. Obviously, increments in cell mass should not be interpreted to mean that cells are at the exponential phase of growth. Biochemists who need large cell yields in their studies often harvest the cultures well beyond the end of the exponential growth, a fact that may explain some of the discrepancies that are noted later in this chapter.

Dissimilatory Reactions

GLUCOSE AND GLYCEROL METABOLISM

Although the mycobacteria can utilize glucose as a carbon and energy source, it is well established that glycerol is the preferred oxidizable substrate for growth of these organisms. The pathways of glucose and glycerol dissimilation can be inferred by identifying key enzymes in known pathways and by determining the distribution of ^{14}C in products from the utilization of the substrate labeled in specific positions (*87*). The use of both methods in the study of the glucose and glycerol dissimilation showed that the mycobacteria utilize the hexose diphosphate pathway (Embden-Meyerhoff pathway, EMP) and the hexose monophosphate pathway (pentose cycle, Warburg-Dickens pathway, HMP).

In entering the cell by diffusion, glucose is converted to glucose-6-phosphate (glucose + ATP hexokinase, Mg^{++} glucose-6- Ⓟ + ADP). Glucose-6- Ⓟ is metabolized either by the EMP pathway or the HMP pathway (*49,64,85*). These pathways are illustrated in Figure 1. Most reactions of the EMP and the HMP pathways were identified in cell-free extracts of the mycobacteria. When cells are incubated in the presence of glucose labeled in position 1 or 6, the distribution of the label in the CO$_2$ produced, in lipid and other cell constituents, was found to be consistent with the functional existence of both pathways (*13,63,64*).

The initial step of glycerol oxidation appears to proceed along two pathways. Either phosphorylation or oxidization may occur first. It appears that the simultaneous existence of the two pathways depends on the age of the cultures or the growth conditions. Thus, Goldman (*27*) reported that α-glycerol- Ⓟ dehydrogenase could not be demonstrated in cell-free extracts of the tubercle bacilli, but the enzyme was demonstrated by Winder and Brennan (*86*) in *M. bovis* BCG and by Hunter (*35*) in *M. butyricum.* To be functional in cells grown in media containing glycerol as the sole carbon and energy source, the triose phosphates are probably converted to glucose-6- Ⓟ. Therefore, as suggested by Goldman, the metabolism of glycerol in the tubercle bacilli may be similar to that of *Aerobacter suboxydans.*

METABOLISM OF PYRUVATE

As shown in Figure 1, pyruvate is a common product in the dissimilation of glucose and glycerol. The mycobacteria are stricly aerobic organisms that meet their energy requirements by the complete oxidation of pyruvate to carbon dioxide and water. Pyruvate is first converted into an activated derivative of acetic acid (acetyl-Coenzyme A). A pyruvate dehydrogenase system that catalizes the oxidative decarboxylation of pyruvate to acetyl-CoA and CO$_2$ was isolated from cell-free extracts of *M. tuberculosis.* The conversion of pyruvate to acetyl-CoA proceeds along a series of complex reactions, some of which were resolved in the tubercle bacilli (*26*).

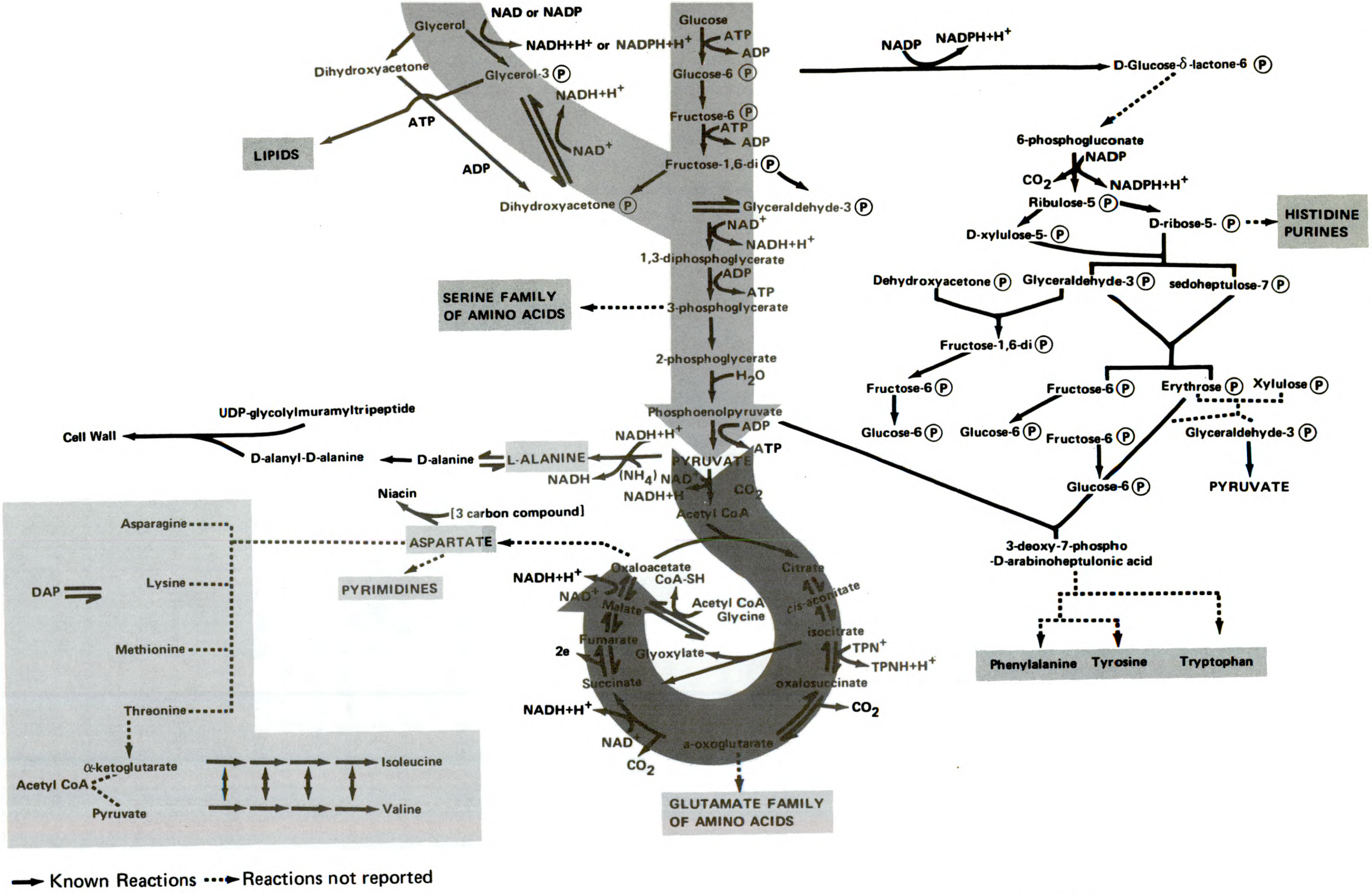

FIGURE 1. Schematic representation of the intermediary metabolism in the mycobacteria.

Acetyl-CoA is transferred to oxaloacetate (a 4-carbon dicarboxylic acid) to form citrate (a 6-carbon dicarboxylic acid). Free Coenzyme A is regenerated in this reaction and can be reutilized in the formation of acetyl-CoA.

The formation of acetyl-CoA and the transfer of the acetyl group to oxaloacetate constitute the first steps in the complete oxidation of pyruvate. The series of reactions that convert pyruvate to carbon dioxide and water is called the Krebs cycle, citric acid cycle or tricarboxylic acid cycle (TCA). The enzymes catalyzing the reactions of the TCA cycle were all identified in cell-free extracts of various mycobacteria.

The electrons removed from substrates during the TCA cycle are carried to molecular oxygen through a series of organized reactions that constitute the terminal respiratory chain. On the other hand, the TCA cycle provides oxoglutarate, which is a precursor for glutamate, and oxaloacetate which is a precursor for aspartate and other amino acids. The removal of oxoglutarate and oxaloacetate from the cycle for biosynthetic processes requires the replenishing of the system. This problem appears to be solved in the mycobacteria by a by-pass in which isocitrate, instead of being converted to oxalosuccinate, is cleaved into succinate and glyoxylate (*26*). This by-pass, called the glyoxylate cycle, replenishes the TCA cycle with the necessary 4-carbon dicarboxylic acids. It should be noted that the enzymes of the cycle are present in cell-free extracts of the mycobacteria grown in glucose or glycerol, but they are functional in *E. coli* only when these organisms are grown in acetate (*81a*).

For each cycle of oxidation, three dehydrogenation steps are linked to NAD, thus providing three molecules of NADH + H^+, whereas the pair of electrons removed from succinate are accepted by the active group of a succinate dehydrogenase. The electrons furnished in these reactions are carried out to molecular oxygen which is reduced to water, as described below.

Aerobic Respiration — the Respiratory Chain

The mycobacteria are strict aerobes, and therefore molecular oxygen must be available as the final electron acceptor. Almost all of the respiratory components of these organisms are membrane bound, and three distinct respiratory chains have been described (Figure 2).

In one chain, the particulate NADH-oxidase couples the oxidation of NADH + H^+ with the reduction of naphthoquinones (Vitamin K_2 in *M. tuberculosis;* Vitamin K_9 in *M. phlei*) through the flavoprotein intermediates (*5,6*). From the quinone, the electrons are transferred to molecular oxygen. In *M. tuberculosis,* the particulate NADH-oxidase is under the control of AMP (*89,90,91*), a compound that is not directly involved in the reaction (allosteric effector).

The oxidation of succinate initiates a second chain. Succinate is converted to fumarate, and the electron acceptor in this reaction is a flavoprotein. The pair of electrons removed from succinate are accepted by an unknown and light-sensitive quinone. The enzyme system that catalyzes the transfer of electrons in this chain through the cytochromes is particulate (membrane bound) and is called the succinic oxidase system. The succinic oxidase system is NAD-independent.

A third chain, also NAD-independent, couples the oxidation of malate to the reduction of Vitamin K_1.

In these respiratory chains, the NAD, flavoproteins and the napththoquinones carry one pair of electrons at a time, whereas the cytochromes can carry only one electron at a time. Thus, the cytochromes have to react twice, and in the series of oxidation-reduction reactions the cytochrome that donates an electron is the reducing agent and the one that accepts it is the oxidizing agent. The reduction of molecular oxygen to water requires the flow of two pairs of electrons ($O_2 + 4H^+ \rightarrow 2H_2O$), and hydrogen peroxide is formed instead of water if only one pair of electrons reacts with molecular oxygen ($O_2 + 2H^+ \rightarrow H_2O_2$).

The above chains are represented in Figure 2, and the sites of oxidative phosphorylation (synthesis of ATP[1]) are indicated. A fourth, nonphosphorylative pathway of oxidation, was also demonstrated. This system was found in *M. phlei,* and it couples the oxidation of NADH + H+ directly to oxygen. This nonphosphorylative chain of oxidation may account for the low P/O ratios (equivalents of phosphate esterified per atom of oxygen taken up) that were found in these organisms.

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[1] *Oxidative phosphorylation* — The free energy of hydrolysis of ATP, in which only one phosphate is hydrolyzed (ATP → ADP+P) is -7000 cal/mole at pH 7.0 and 25°C. For various reasons, the free energy of hydrolysis under intracellular conditions may be higher (-12,000 cal/mole). Thus, the synthesis of ATP from ADP requires at least this much energy. As the electrons pass from one acceptor to the next in the respiratory chain, there is a stepwise release of energy (total: -52,000 cal/mole). The sites of oxidative phosphorylation in the chain are thermodynamically equivalent to ATP, and the respiratory chain functions as the mechanism whereby the energy released from the dissimilation of nutrients is stored in usable form. ATP is the most important link between the energy yielding and the energy requiring reaction in the cell.

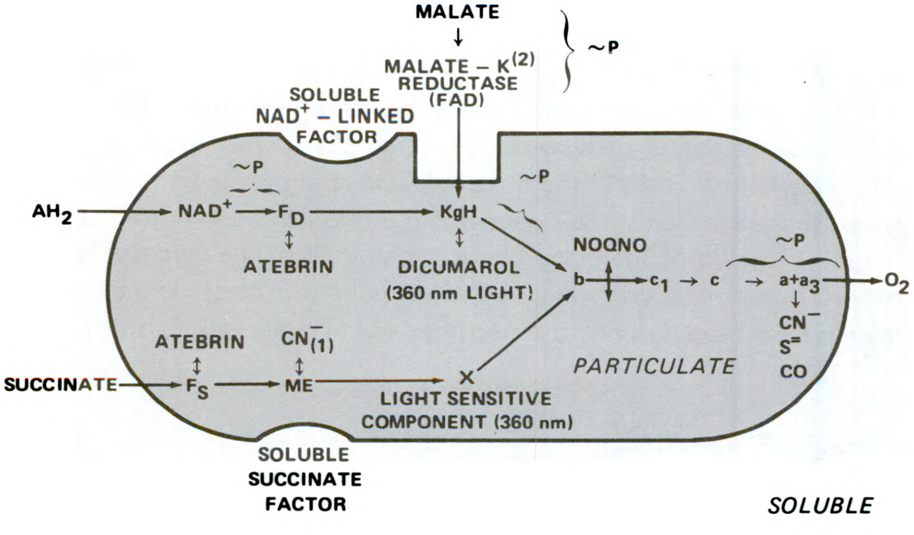


FIGURE 2. Soluble and particulate respiratory chains of *M. phlei* (adapted from Asano and Brodie, 5). K = Vitamin K; NOQNO = 2-n-nonylhydroxyquinoline N-oxide; (1) = order of metal in sequence unknown; (2) = also in *M. avium.*

The disposal of hydrogen peroxide that may accumulate can be considered a final reaction of the terminal respiratory chain. This is accomplished in the mycobacteria by two iron-porphyrin enzymes (catalase and peroxidase), which occur in all the mycobacteria. Catalase catalyzes the reaction $H_2O_2 + H_2O_2 \rightarrow O_2 + 2H_2O$. The enzyme is found in the non-particulate, soluble fraction of cell-free extracts of the mycobacteria — *M. tuberculosis, M. smegmatis* and *M. phlei* (*71*). Studies with whole cells indicated that more than one kind of catalase in respect to heat stability was present in the mycobacteria (*45,70a*). The catalase isoenzymes were separated by electrophoresis in agarose plates (*30*), and acrylamide gel electrophoresis (*7*). By acrylamide gel electrophoresis, which separates molecules on the basis of electrical charge and molecular weight, five catalase isoenzymes were identified in *M. terrae, M. triviale* and *M. smegmatis*; one is heat sensitive a second is partially inactivated by heat, and three are heat resistant.

Peroxidase catalyzes the reaction donor + $H_2O_2 \rightarrow$ oxidized donor + H_2O. The enzyme is also found in the soluble fraction of cell-free extracts. According to Winder (*84*), the peroxidatic activity is due to catalase, since no evidence has been found that a true peroxidase exists in *M. smegmatis* and *M. tuberculosis*.

Biosynthesis of Small Molecules

INTRODUCTION

The carbon skeleton of the amino acids and the purine and pyrimidine bases is furnished by glucose or glycerol. It was shown earlier that the dissimilation of the carbon source appears to follow two pathways: the EM pathway and the HMP pathway. In the dissimilation of glucose, and probably also of glycerol, both pathways are functional. About two-thirds of the glucose is metabolized by the EM and about one-third by the HMP pathway. As shown in Figure 1, these pathways can supply all the carbon units for the biosynthesis of the amino acids, the purine and pyrimidine bases and the fatty acids.

Not much is known of the biosynthetic capabilities of the mycobacteria, although one may infer these to be many, as it might be expected in prototrophic organisms. So far, only a few pathways have been described: biosynthesis of alanine, valine, isoleucine and the fatty acids.

BIOSYNTHESIS OF ALANINE

Alanine is formed from pyruvate which is also a precursor of valine and isoleucine. These three amino acids constitute the pyruvate family of amino acids.

In the bacteria, alanine can be formed by transamination with glutamate or directly by the reductive amination of pyruvate. An alanine dehydrogenase was purified from cell-free extracts of the tubercle bacilli (*26*). It is $(NADH + H^+)$ dependent and catalyzes the reaction $CH_3 \bullet CO \bullet COO^- + NH_4 + (NADH + H^+) \rightleftarrows (CH_3 \bullet CHNH_2)\ COO^- + NAD^+ + H_2O$.

BIOSYNTHESIS OF VALINE AND ISOLEUCINE

The biosynthesis of these two amino acids proceeds through a series of parallel reactions catalyzed by four common enzymes. In *M. tuberculosis,* the conversion of the 2-acetohydroxy acids to the correspondent 2 β-dihydroxy acids may proceed by an alternate route (*1,2*) as shown in Figure 3.

BIOSYNTHESIS OF THE AROMATIC AMINO ACIDS

The three aromatic amino acids (phenylalanine, tyrosine and tryptophan) are derived from a common precursor compound, 3-deoxy-7-phospho-D-arabinoheptulosonate. This compound arises by the condensation of D-erythrose-4-phosphate with phosphoenolpyruvate. The enzyme that catalyzes this condensation reaction has been found in cell-free extracts of *M. avium, M. phlei* and *M. smegmatis* (*39*). The other enzymes in this complex and branched pathway have not been described in the mycobacteria.

OTHER AMINO ACIDS AND THE PURINE AND PYRIMIDINE BASES

No data is available in the literature on the biosynthesis of small molecules other than those described above and the fatty acids.

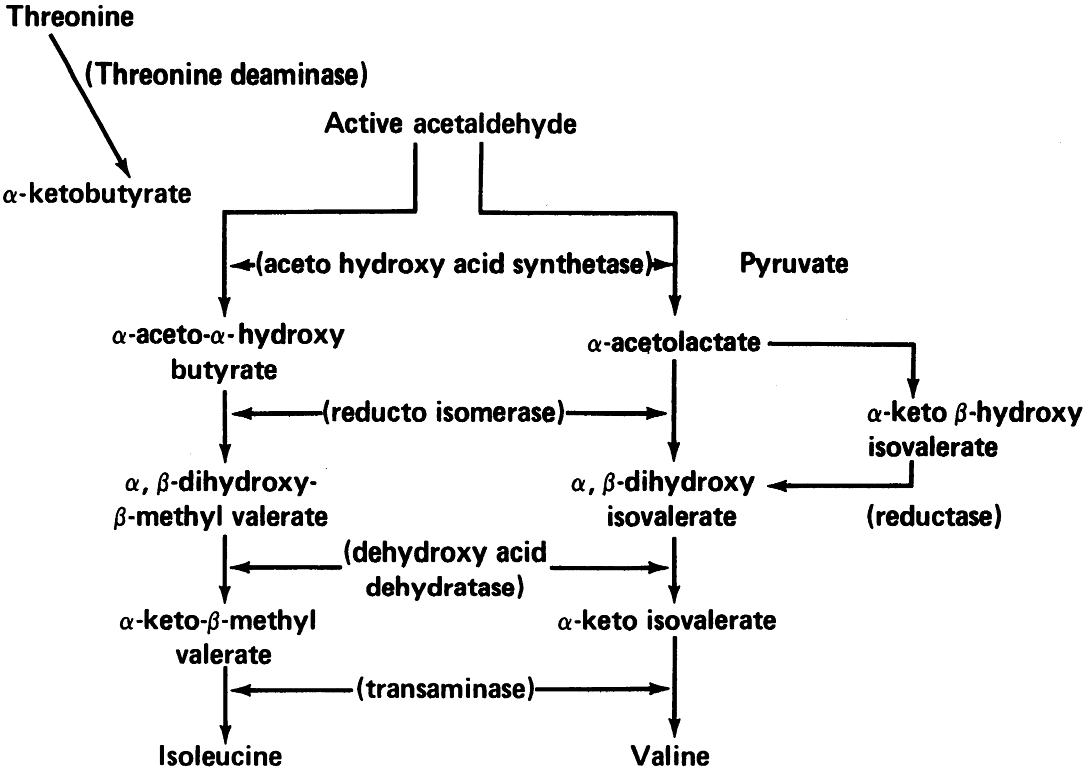
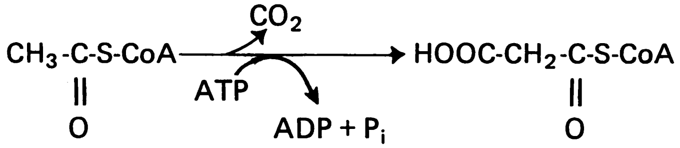


FIGURE 3. Biosynthesis of valine and isoleucine in *M. tuberculosis* (adapted from Allaudeen and Ramakrishnan, *1*).

Biosynthesis of Lipids

BIOSYNTHESIS OF THE FATTY ACIDS

The ultimate source of carbon in the fatty acids is acetyl-CoA, and the first reaction if the carboxylation of acetyl-CoA to form malonyl-CoA:



$$CH_3\text{-}\underset{\underset{O}{\|}}{C}\text{-}S\text{-}CoA \xrightarrow[ATP \qquad ADP + P_i]{CO_2} HOOC\text{-}CH_2\text{-}\underset{\underset{O}{\|}}{C}\text{-}S\text{-}CoA$$

The malonyl CoA is the precursor of all fatty acids whose synthesis takes place in the soluble fraction of cytoplasm. Acetyl-CoA and malonyl CoA condensate with the release of CO_2. These are the initial reactions (a total of seven) catalyzed by the multienzyme fatty acid synthetase system. The fatty acids appear to elongate by successive additions of malonyl-S-acyl carrier protein. Evidence for the elongation mechanism via malonyl-CoA was given by Goldman and his associates (*40,65*).

BIOSYNTHESIS OF THE MANNOPHOSPHOLIPIDS

The information available regarding the metabolism of the phospholipids in the mycobacteria is scarce. During investigations on the variation in the lipid content of *M. tuberculosis* under different growth conditions Creighton, Chang and Anderson (*14*) reported that the phosphatides were undetectable in cells of *M. tuberculosis* grown in a synthetic medium in which glucose was the only source of carbon. This observation is now known to be incorrect.

Hill and Ballou (*34*) suggested that, in *M. phlei,* the biosynthesis of the mannophospholipids proceeds by the transfer of mannose from guanosine diphosphate mannose (GDP-mannose) to phosphatidyl *myo*-inositol, the higher numbers of the series of the oligomannosides being found by the stepwise addition of mannose to the acceptor.

By adding *myo*-inositol-2-^{3}H to *M. phlei* in the late logarithmic phase of growth for 2 hours, Hill and Ballou (*34*) verified that most of the specific radioactivity was incorporated into phosphotidyl-*myo*-inositol. This observation suggested that this compound was a precursor of the oligomannoside derivatives. Furthermore, they showed that *in vitro* the radioactivity from GDP-mannose-^{14}C was incorporated into the phospholipids present in a cell free fraction of *M. phlei.* In *M. tuberculosis* the transfer of radioactivity from GDP-mannose-1-^{14}C was catalyzed by particulate enzymes (*76,77*), which appeared to be identical to those of *M. phlei.* A polyisoprenoid appears to be involved in the transfer of the sugar moieties to an acceptor (*78*).

BIOSYNTHESIS OF THE CAROTENOID PIGMENTS

It is generally accepted that mevalonic acid is the immediate precursor in the biosynthesis of these isoprenoid lipids. Mevalonic acid ($C_6 H_{12}O_4$) is synthesized from acetate and is converted into geranylgeranyl pyrosphosphate. The first carotenoid, phytoene, is then formed by the dimerization of geranylgeranyl pyrophosphate (*28a*).

Phytoene is converted into lycopene in a series of dehydrogenation steps; lycopene, an acyclic carotenoid, is then cyclicised to form the monocyclic and then the dicyclic carotenoids.

Whole cells of *M. phlei* and *M. kansasii* (*20,69a*) incorporate ^{14}C-acetate very rapidly in the carotenoid pigments. Acetate -2 -^{14}C was better than acetate-1-^{14}C as a substrate, because more respiratory $^{14}CO_2$ is released from the latter. Attempts to label the carotenoids of *M. kansasii* using mevalonic acid-2-^{14}C were unsuccessful (*20*), perhaps because the cells are impermeable to the substrate.

The enzymatic reactions that were demonstrated in cell free extracts of a photochromogenic *Mycobacterium* sp. (*67b*) are indicated in Figure 4. Other enzymatic reactions were not directly demonstrated; however, the carotenoids isolated and characterized so far suggest that the pathway in Figure 5 is common in the mycobacteria as it is in other bacteria. Studies using known inhibitors of carotene synthesis, such as diphenylamine (*67a*) and nicotine (*34a*), yielded data consistent with the hypothetic pathway in *M. marinum* (*10*) and in *M. kansasii* (*19*). Further evidence was obtained in the study of the carotenoids that accumulate in strains of *M. kansasii* carrying genetic blocks at different sites (*19*).

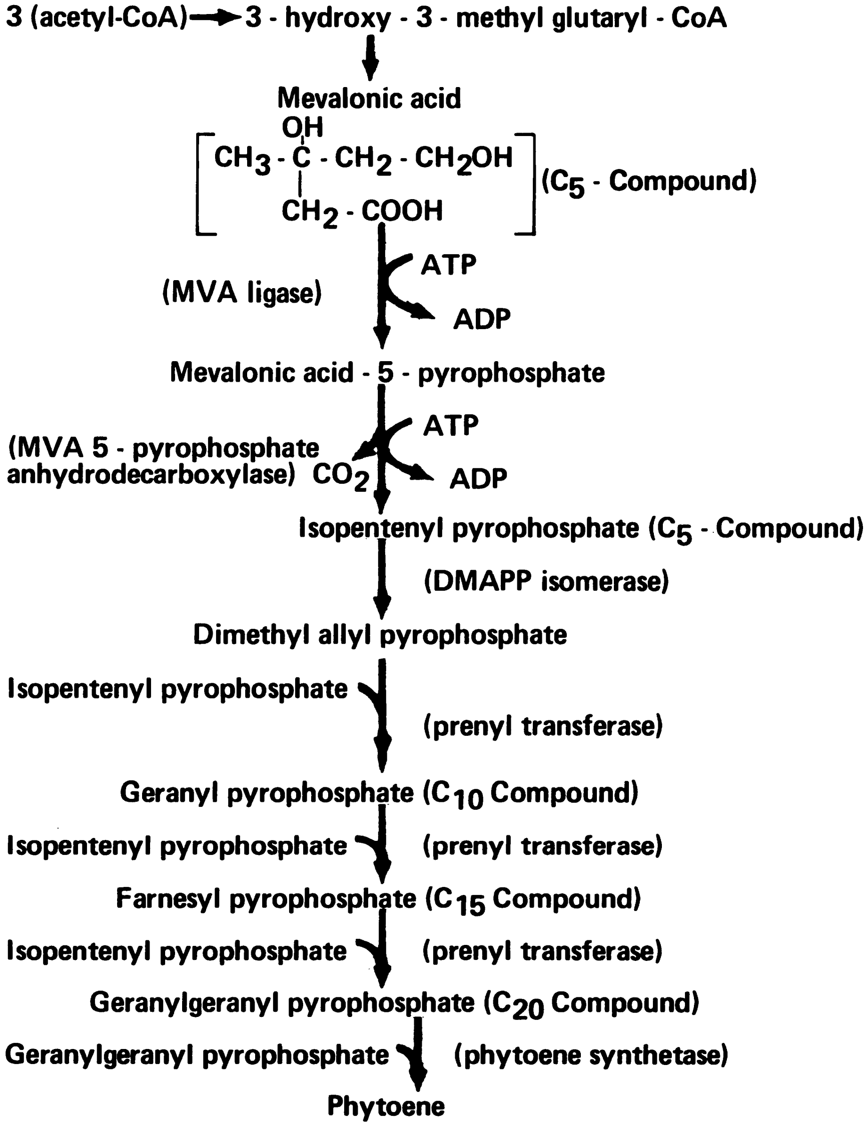
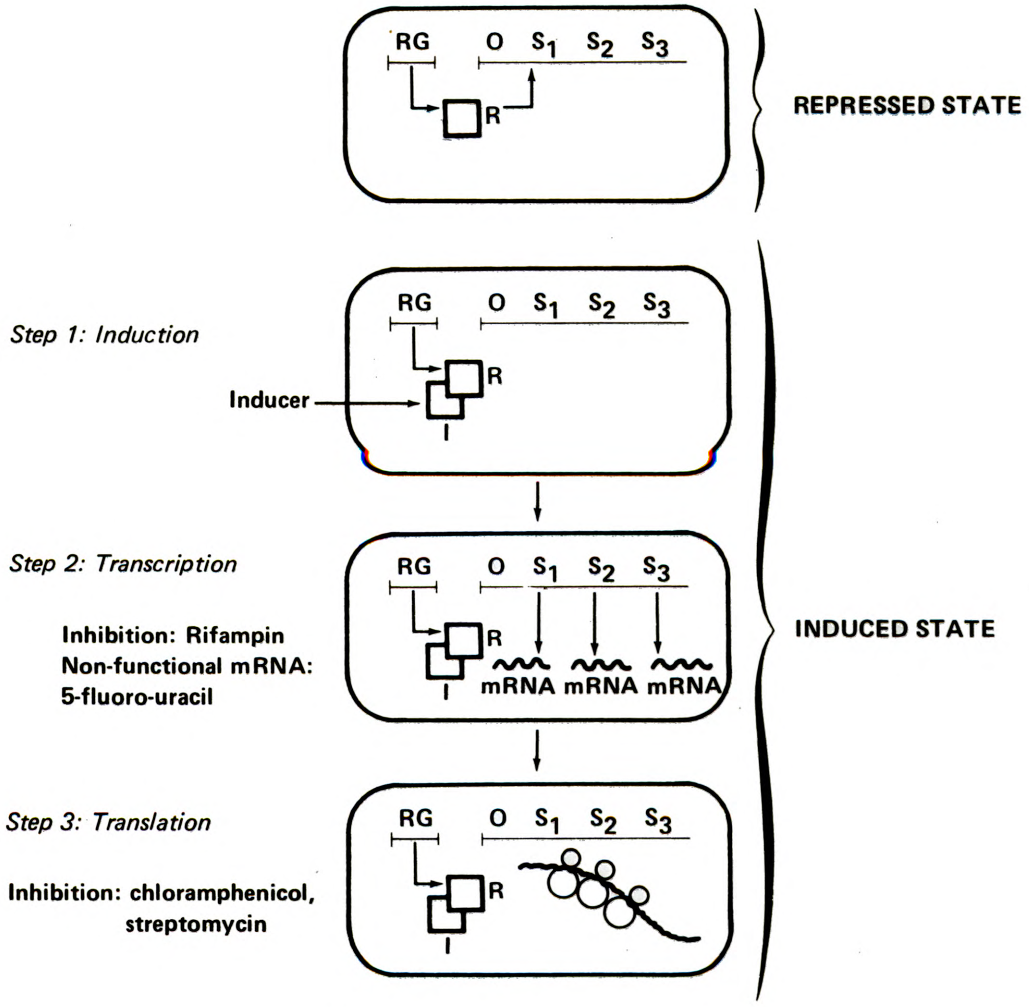

3 (acetyl-CoA) → 3 - hydroxy - 3 - methyl glutaryl - CoA
Mevalonic acid
OH
CH3 - C - CH2 - CH2OH
CH2 - COOH
(C5 - Compound)
(MVA ligase)
ATP
ADP
Mevalonic acid - 5 - pyrophosphate
(MVA 5 - pyrophosphate anhydrodecarboxylase)
CO2
ATP
ADP
Isopentenyl pyrophosphate (C5 - Compound)
(DMAPP isomerase)
Dimethyl allyl pyrophosphate
Isopentenyl pyrophosphate
(prenyl transferase)
Geranyl pyrophosphate (C10 Compound)
Isopentenyl pyrophosphate
(prenyl transferase)
Farnesyl pyrophosphate (C15 Compound)
Isopentenyl pyrophosphate
(prenyl transferase)
Geranylgeranyl pyrophosphate (C20 Compound)
Geranylgeranyl pyrophosphate
(phytoene synthetase)
Phytoene

FIGURE 4. Biosynthesis of phytoene (*67b*).

Biosynthesis of Macromolecules

Very little is known about the biosynthesis of macromolecules in the mycobacteria. However, there is some data on the synthesis of the following: protein in cell-free extracts, the cell-wall peptidoglycan, and messenger-RNA. The information that is available on protein synthesis was mentioned in relation to the ribosomes (Chapter 2), synthesis of the peptidoglycan was mentioned in relation to the cell-wall structure (Chapters 3 and 5), and synthesis of mRNA is mentioned in Chapter 5 in relation to the mode of action of the antibiotic rifampin.



RG = regulatory gene
O = operator
S_1, S_2, S_3 = structural genes
R = repressor
I = inducer
I-R = inducer-repressor non-functional complex

FIGURE 5. Schematic representation of inducible enzyme synthesis and level of action of some inhibitors.

Control Mechanisms

INTRODUCTION

In a growing cell the metabolic pathways must be coordinated so that it can adjust rapidly to changes in the growth conditions. It was noted before that the mycobacteria are able to grow in simple media containing glucose or glycerol as the only carbon source and energy, and that nitrogen can be supplied by a simple salt like ammonium chloride. In a simple medium if the concentration of carbon or of nitrogen are made variable, the cell mass formed is proportional to the concentration of the substrate (*31*). This relationship

$$Y = \frac{\text{cell mass (mg dry weight)}}{\text{substrate utilized (mg)}}$$

indicates that growth is limited by reducing the concentration of an essential substrate or metabolite (growth limiting factor). As the concentration of the growth limiting factor is increased, so does the growth rate, the cellular concentration of protein, RNA and DNA, and the size of the cells. Thus, at a given temperature the cells tend to grow faster and to be larger in a rich medium than in a poor medium. When cultures on balanced growth in a rich medium are shifted to a poor medium ("shift down"), the synthesis of RNA and the increase in cell mass stop immediately, whereas cell division and DNA synthesis continue at the old rate for some time. When the shift is from a poor to a rich medium ("shift up"), the rate of synthesis of RNA and the cell mass increase immediately, whereas DNA synthesis and cell division continue at the old rate (*52,69*). The responses described above are manifestations of the regulatory mechanisms the bacteria are endowed with and show that transitions from one physiological state to another are preceded by a period of adjustment to the conditions present in the new environment (*43,44*).

Patterns of metabolic control such as these mentioned above, have not been satisfactorily investigated in the mycobacteria. Yet, the study of the ability of the mycobacteria to reversibly shift from one physiological state to another in reference to changes in the environment, and particularly, investigations of the underlying mechanisms in biochemical terms may have important practical consequences. For instance, although *M. tuberculosis* are prototrophic organisms their isolation from pathologic secretions is unsuccessful when complex media are used. Clearly, *M. tuberculosis* may not be able to change from one physiological state to another and, consequently, the cells die (*18*). Another example of undifferentiated control is that, under conditions of starvation, mycobacterial cells utilize lipid and glycogen stored while growing in the presence of an excess of glucose and glycerol; under these conditions, glycogen is utilized first, which suggests a regulatory mechanism still to be clarified (*79,80*).

In addition to the crude overall control that is established, or imposed upon the cells, only because the metabolic pathways are related to each other (*44*) and are precisely adjusted to each other, the cells evolved specific control mechanisms that allow them to respond rapidly to environment changes. These mechanisms are inductive, repressive and feed back inhibition of the protein enzymes. (For a recent review, see reference *53*).

INDUCTION AND REPRESSION

Certain enzymes are formed only in the presence of the respective substrate. Monod (*60*) investigated this phenomenon which he referred to as induction. Monod and Jacob

(*37,61*) demonstrated that the enzymes are formed *de novo* from amino acids and that the inducible systems are genetically controlled. Figure 5 is a generalized view of inducible systems. The scheme, based on the Jacob and Monod model (*37*), shows that the inducer enters the cell and reacts with a repressor whose function is to keep the information transfer "cut off." When the inducer (effector) binds to the repressor, a small molecular weight protein (*25*), the latter is rendered nonfunctional. Consequently, the information transfer is "turned on" (*61*). The first step in the transfer of the genetic information is the transcription of the genetic code into messenger RNA (*29*) which is followed by its translation on the ribosomes (*12*) where the polypeptide chains are synthesized. Thus, the inducible enzyme synthesis progresses in three main steps: induction (reaction between the effector and the repressor), transcription (synthesis of mRNA) and translation (synthesis of the polypeptides).

The remarkable specificity of the inducible systems was noted very early by Monod (*60*). In other words, only the enzyme that acts upon the substrate is synthesized; the synthesis of unrelated enzymes is not induced. The nature of the link between the inducer, the repressor, and the operator is still unclear.

The induction step occurs almost instantaneously, while the transcription and the translation steps start in this order after a short lag. On the other hand, the synthesis of mRNA stops immediately after the inducer is withdrawn but protein synthesis may continue for a while. Since mRNA has a half life of 2 minutes or less, protein synthesis cannot continue indefinitely and stops when the concentration of mRNA reaches a certain low value. Kinetic studies showed that the rate of enzyme synthesis is proportional to the amount of mRNA present (*41*). According to the model, inhibitors of protein synthesis must inhibit inducible systems. Furthermore, the transcription and translation steps may be dissociated by inhibitors that specifically block one or the other.

The system that was studied in more detail in the mycobacteria is the induction of carotenogenesis in *M. marinum* and *M. kansasii.* Both synthesize large amounts of β-carotene on exposure to visible light. The mechanism of photochromogenecity in these bacteria was shown to consist of two reactions: the first reaction is photooxidative and the second is an inducible reaction.

The photooxidative reaction requires oxygen but is independent of temperature, and it exhibits an action spectrum characteristic for each of the organisms. The maxima for *M. marinum* were reported to be 410 nm, 439 nm, 502 nm and 528 nm by Mathews (*55*), but to be 404 nm, 493 nm and 577 nm by Batra and Rilling (*9*); the maxima for *M. kansasii* were 410 nm, 540 nm and 650 nm (*20*); and the maxima for an unidentified mycobacterium were 365 nm and 460 nm (*9*). The substrate of the photooxidative reaction was postulated to be either a repressor that is rendered nonfunctional or a compound that is converted into an inducer (*10*).

The induction reaction is dependent on oxygen and temperature. Kinetic studies in *M. kansasii* showed that mRNA synthesis starts almost immediately after exposure of the cells to light, while the synthesis of the carotenoids begins after a lag of approximately 2 minutes. Contrary to the usual stop of mRNA once the inducer is withdrawn, the synthesis of mRNA in cells shifted from light to darkness continued for about 40 to 50 minutes. As expected for inducible systems, carotenogenesis is blocked by inhibitors of protein synthesis such as chloramphenicol, rifampin, streptomycin and puromycin (*10,20*). A comprehensive review on the mechanisms of photochromogenicity was published by Batra recently (*10*).

The Jacob and Monod model predicts that an inducible system can be converted into a constitutive system by mutations in the regulator gene. The finding of *M. kansasii* mutants constitutive in respect to carotenogenesis (*75*), is further evidence to support the conclusion that the induction of carotenogenesis in *M. kansasii* follows closely the Jacob and Monod model. Furthermore, the isolation of a mutant that accumulates lycopene (an intermediate in β-carotene synthesis) on induction showed that mutations in the structural genes are distinct from the mutations in the regulator gene (19).

FEED-BACK INHIBITION

The enzymatic reactions in the amino acids and nucleotides biosynthetic pathways are controlled by feed-back inhibition (end-product inhibition) and repression. Usually, the end-product specificially inhibits the first enzyme in its biosynthetic pathway. Because the end-product is structurally very different from the substrate of the first enzyme, the enzyme must have two recognizing sites: the active site that recognizes the substrate and a site (allosteric site) that recognizes the inhibitor (effector). In the mycobacteria, the only enzyme that is known to be controlled by end-product inhibition is 3-deoxy-D-arabino-heptulozonate-7-phosphate. Five patterns of control of this enzyme are known: isoenzyme feed-back inhibition (*74*); cumulative feed-back inhibition (*88*); concerted feed-back inhibition (*16*); sequential feed-back inhibition (*38*); and single effector feed-back inhibition (*38,39*). The enzyme in *M. avium, M. phlei* and *M. smegmatis* was reported to be inhibited by tyrosine but not by phenylalanine and tryptophan *in vitro* (Table 3). Therefore, it appears to be controlled by single effector feed-back inhibition. According to Jensen *et al.* (*39*), the growth of organisms in which the control was by single effector enzymes was not inhibited by the exogenous supplement of the amino acid capable of inhibiting the synthetase *in vitro.* These observations are at variance with reports according to which the aromatic amino acids inhibit the growth of tubercle bacilli (*18,54,93*).

TABLE 3. Inhibition (%) of 3-deoxy-D-arabino-heptulozonate-7-phosphate Synthetase of *M. avium, M. phlei,* and *M. smegmatis* and Inhibition of Growth (%) of *M. tuberculosis* by the Aromatic Amino Acids [adapted from references *38* and *39*]

	% Inhibition				
Species	**Tyr.**	**Phen.**	**Tryp.**	**ARO[1]**	**TPT[2]**
M. avium	50	0	17	70	- -
M. phlei	29	0	3	33	- -
M. smegmatis	88	0	10	92	- -
M. tuberculosis	25	26	57	- -	52

[1] ARO = mixture of tyrosine, phenylalamine and tryptophan at 0.35 mM and p-amino-benzoate and p-hydroxybenzoate at a 10-fold lower concentration.

[2] TPT = mixture of tyrosine, phenylalamine and tryptophan at 1 mM concentration.

REFERENCES

1. Allaudeen, H. S. and Ramakrishnan, T. (1968) Arch. Biochem. Biophys. 125:199.

2. Allaudeen, H. S. and Ramakrishnan, T. (1970) Arch. Biochem. Biophys. 140:245.

3. Antoine, A. D. and Tepper, B. S. (1969) J. Gen. Microbiol. 55:217.

4. Antoine, A. D. and Tepper, B. S. (1969) J. Bacteriol. 100:538.

5. Asano, A. and Brodie, A. F. (1964) Biochem. Biophys. Res. Comm. 15:50.

6. Asano, A. and Brodie, A. R. (1965) J. Biol. Chem. 240:4002.

6a. Asano, A. and Brocie, A. R. (1964) J. Biol. Chem. 239:4280.

7. Bartholomew, W. R. (1968) Amer. Rev. Resp. Dis. 97:710.

8. Bastarrachea, F., Anderson, D. G. and Goldman, D. S. (1961) J. Bacteriol. 82:94.

9. Batra, P. P. and Rilling, H. C. (1964) Arch. Biochem. Biophys. 107:485.

10. Batra, P. P. (1971) Photophysiology 6:47.

11. Brennan, P. and Ballou, C. E. (1968) J. Biol. Chem. 243:2975.

12. Brenner, S., Jacob, F. and Meselson, M. (1961) Nature 100:576.

13. Clifton, C. E. (1961) Amer. Rev. Resp. Dis. 84:705.

14. Creighton, M. M., Chang, L. H. and Anderson, R. J. (1944) J. Biol. Chem. 154:569.

15. Darzins, E. (1958) The Bacteriology of Tuberculosis, University of Minnesota Press, Minnesota.

16. Datta, P. and Gest, H. (1964) Proc. Nat. Acad. Sci., Washington, 52:1004.

17. David, H. L. (1968) M. S. Thesis, University of Wisconsin, Madison, Wisc.

18. David, H. L. (1972) Amer. Rev. Resp. Dis. 105:944.

18a. David, H. L. (1973) Amer. Rev. Resp. Dis. 108:1175.

19. David, H. L. (1974) Appl. Microbiol. 28:696.

20. David, H. L. (1974) J. Bacteriol. 119:527.

21. Drea, W. and Andrejew, A. (1953) The Metabolism of the Tubercle Bacilli, Charles C. Thomas, Pub., Springfield, Ill.

22. Dunphy, P. G., Gutnick, D. L., Phillips, P. G. and Brodie, A. F. (1968) J. Biol. Chem. 243:398.

23. Edson, N. L. (1951) Bacteriol. Rev. 15:147.

24. Fischer, M. W., Kirchheimer, W. F. and Hess, A. R. (1951) J. Bacteriol. 62:319.

25. Gilbert, W. and Muller-Hill, B. (1966) Proc. Natl. Acad. Sci., Washington, 56:1891.

26. Goldman, D. S. (1961) Adv. Tuberc. Res. 11:1.

27. Goldman, D. S. (1963) J. Bacteriol. 86:30.

28. Goldman, D. S. and Wagner, M. J. (1962) Biochem. Biophys. Acta 65:297.

28a. Goodwin, T. W. (1971) in "Carotenoids" edited by O. Isler, Birkhauser Verlag, Basel and Stuttgart, page 577, 1971.

29. Gnos, H., Hiatt, H., Gilbert, W., Kirland, C. G., Risebrough, R. W. and Watson, J. D. (1960) Nature 190:581.

30. Gruft, H. and Gaafar, H. A. (1971) J. Chromat. 56:168.

31. Gunsalus, I. C. and Shuster, C. W. (1961) in "The Bacteria: A Treatise on Structure and Function," Vol. II, Academic Press, New York.

32. Gutierrez-Vasquez, J. M. (1956) Amer. Rev. Tuberc. 74:50.

33. Halpern, B. and Kirchheimer, W. F. (1954) Amer. Rev. Tuberc. 70:665.

34. Hill, D. L. and Ballou, C. E. (1966) J. Biol. Chem. 241:895.

34a. Howes, C. D. and Batra, P. P. (1970) Biochem. Biophys. Acta 222:174.

35. Hunter, G. J. E. (1953) Biochem. J. 55:320.

36. Indira, M. and Ramakrishnan, T. (1963) Amer. Rev. Resp. Dis. 88:509.

37. Jacob, F. and Monod, J. (1961) J. Mol. Biol. 3:318.

38. Jensen, R. A. and Nester, E. W. (1965) J. Mol. Biol. 12:468.

39. Jensen, R. A., Nester, D. S., and Nesser, E. W. (1967) J. Bacteriol. 94:1582.

39a. Johnson, J. H., Reed, B. C. and Rilling, H. C. (1974) J. Biol. Chem. 249:402.

40. Kanemasa, Y. and Goldman, D. S. (1965) Biochim. Biophys. Acta 98:476.

41. Kepes, A. (1963) Biochim. Biophys. Acta 76:293.

42. Kjeldgaard, N. O., Maale, O. and Schaechter, M. (1958) J. Gen. Microbiol. 19:608.

43. Kjeldgaard, N. O., Maale, O. and Schaechter, M. (1958) J. Gen. Microbiol. 19:607.

44. Kornberg, H. L. (1965) XV Symposium of the Society of General Microbiology, Cambridge Univ. Press. p. 8.

45. Kubica, G. P. and Pool, G. L. (1960) Amer. Rev. Resp. Dis. 81:387.

46. Kull, F. C. and Grimm, M. R. (1952) J. Bacteriol. 64:431.
47. Kusunose, E. and Goldman, D. S. (1963) Biochim. Biophys. Acta 73:391.
47a. Kushawa, S. C., Suzue, C., Subbarayan, C. and Porter, J. W. (1970) J. Biol. Chem. 245:4708.
48. Kusunose, M., Nagai, S., Kusunose, E., and Yamamura, Y. (1956) J. Bacteriol. 72:754.
49. Long, E. R., Anderson, R. J., Rittenberg, D., Karnovsky, M. L. and Henderson, H. J. (1955) Amer. Rev. Tuberc. 71:609.
50. Long, E. (1958) The Chemistry and Chemotherapy of Tuberculosis, the Williams & Wilkins Company, New York.
51. Lyon, R. H., Lichstein, H. C. and Hall, W. H. (1961) Amer. Rev. Resp. Dis. 83:255.
52. Maale, O. (1961) Cold Spring Harbour Symp. Quant. Biol. 26:45.
53. Mandelstam, J. and McQuillen, K. (1968) Biochemistry of Bacterial Growth, John Wiley & Sons, Inc., New York.
54. Marshak, A. (1951) J. Bacteriol. 61:1.
55. Matthews, M. M. (1963) Photochem. Photobiol. 2:1.
56. McLaren, C. L. and Jann, G. J. (1955) Amer. Rev. Tuberc. 71:260.
57. Merrill, M. H. (1930) J. Bacteriol. 20:235.
58. Merrill, M. H. (1931) J. Bacterol. 21:231.
59. Miller, J. L. and Roessler, W. G. (1956) Amer. Rev. Tuberc. 73:716.
60. Monod, J. (1955) Exposes Ann. Biochem. Medicale 17:195.
61. Monod, J., Jacob, R. and Gros, F. (1961) Biochem. Soc. Symp. 21:104.
62. Nester, E. W. and Jensen, R. A. (1966) J. Bacteriol. 91:1594.
63. Noble, R. E., Koch-Weser, D. and Noble, E. P. (1962) Amer. Rev. Resp. Dis. 86:391.
64. O'Barr, T. P. and Rothlauf, M. V. (1970) Amer. Rev. Resp. Dis. 101:964.
65. Pierard, A. and Goldman, D. S. (1963) Arch. Biochem. Biophys. 100:56.
66. Proskauer, B. and Beck, M. (1894) Zeit. Hyg. und Infekionk. 18:128.
66a. Ramakrishnan, T., Suryanarayana, P. and Gopinathare, K. P. (1972) Bacteriol. Rev. 33:65.
67. Rilling, H. C. (1962) Biochem. Biophys. Acta 60:548.
67a. Rilling, H. C. (1965) Arch. Biochem. Biophys. 110:39.
68. Robson, J. M. and Sullivan, F. M. (1957) Amer. Rev. Tuberc. 75:756.
69. Schaechter, M. (1961) Cold Spring Harbour Symp. Quant. Biol. 26:53.
69a. Schlegel, H. G. (1959) J. Bacteriol. 77:310.
70. Schaefer, W. B., Marshak, A. and Burkhart, B. (1949) J. Bacteriol. 58:549.
70a. Schweiger, O., Roman, E. and Soos, I. (1958) Amer. Rev. Tuberc. 77:146.
71. Segel, W. P. and Goldman, D. S. (1963) Biochem. Biophys. Acta 73:780.
72. Shepard, C. C., McRae, D. H. (1965) J. Bacteriol. 89:365.
73. Snow, G. A. (1970) Bacteriol. Rev. 34:100.
74. Stadman, E.R. (1966) Adv. Enzymol. 28:41.
75. Tacquet, A., Tison, F. and Devulder, B. (1965) Ann. Inst. Pasteur. 108:514.
76. Takayama, K. and Goldman, D. S. (1967) Fed. Proc. 26:796.
77. Takayama, K. S. and Goldman, D. S. (1969) Biochim. Biophys. Acta 176:196.
78. Takayama, K. and Goldman, D. S. (1970) J. Biol. Chem. 245:6251.
79. Tepper, B. S. (1965) Amer. Rev. Resp. Dis. 92:75.
80. Tepper, B. S. (1968) J. Bacteriol. 95:1713.
81. Volk, W. A. and Myrvik, Q. N. (1953) J. Bacteriol. 66:386.
81a. Wegener, W. S., Reeves, H. C., Rabin, R. and Ajl, S. J. (1968) Bacteriol. Rev. 32:1.
82. Willet, H. P. (1958) Proc. Soc. Exptl. Biol. Med. 99:177.
83. Willet, H. P. (1960) Amer. Rev. Resp. Dis. 81:653.
84. Winder, F. (1960) Amer. Rev. Resp. Dis. 81:68.
84a. Winder, F. (1966) J. Bacteriol. 92:413.
85. Winder, F. G. and O'Hara, C. (1964) Biochem. J. 90:122.
86. Winder, F. G. and Brennan, P. J. (1966) J. Bacteriol. 92:1846.
87. Wood, W. A. (1961) in "The Bacteria," edited by Gunsalus and Stanier, Vol. II, p. 65, Academic Press, New York.
88. Woolfolk, C. A. and Stadman, E. R. (1964) Biochem. Biophys. Res. Comm. 17:313.
89. Worcel, A. and Goldman, D. S. (1964) Biochem. Biophys. Res. Comm. 17:559.
90. Worcel, A. (1966) Biochem. Biophys. Acta 113:178.
91. Worcel, A. and Goldman, D. S. (1967) Arch. Bioderm. Biophys. 118:420.
92. Youmans, G. P. and Youmans, A. S. (1949) J. Bacteriol. 58:427.
93. Youmans, A. S. and Youmans, G. P. (1954) J. Bacteriol. 67:734.

CHAPTER 4
Genetics of the Mycobacteria

Introduction

This chapter is concerned with the hereditary constitution of the mycobacteria (their genotype) as it relates to and determines their observable characteristics (their phenotype). Almost all the literature now available describes the frequency distribution of genotypes that arise from mutation and selection, rather than the nature, mode of replication, or function of the genetic material. Additional information is contributed by studies on the effect ultraviolet light radiation and other mutagens exert on the mycobacteria, and by investigations with the mycobacteriophages.

Genetic Variation

SPONTANEOUS MUTATIONS – DEFINITIONS

Mutations are sudden and heritable changes in the properties of an organism. In genetic terminology, a property under observation is called a character. Thus, an organism may be defined by a large number of characters, and those found in the majority of strains isolated from nature define the wild-type parental organism. For example, the wild-type parental organism *M. tuberculosis* could be conveniently defined as follows: niacin^{+}, cat R$^{\circ +}$, cat pH 7.0/68$^{\circ -}$, Inh^{s}, Str^{s}. The plus sign (+) indicates that the character is present and the minus sign (–) that it is not; in relation to drug sensitivity the superscript *s* means sensitive and *r* means resistant. These characters describe the organism's phenotype. Thus, phenotype can be defined as the identifiable expression of the genetic information (genotype) encoded in the organism's chromosome.

In its simplest form the chromosome, a single molecule of DNA in the bacteria, may be compared to a computer tape in which all the information of the organism is encoded. It is a very special kind of tape, however. Thus, it has the property of making exact replicas of itself which are transferred to the next generation of the organism. Consequently, the stability of the species is guaranteed. The information that is contained in the program is not affected by the environment, and indeed chemical or physical agents that can interact with the chromosomal DNA, and can therefore modify the program, are also highly lethal; among the few survivants a few may have one or more characters changed, and the changed characters will be transferred to their progeny from then on. The manner the program is expressed, on the other hand, is influenced by the environment. For example, the genome of *M. kansasii* contains the information for the cell to make the series of enzymes that are necessary to convert phytoene into beta-carotene. Yet, this information is not utilized unless the cells are exposed to visible light in which case every cell that was exposed begins to synthesize the pigment. The descendants of these cells are not pigmented, unless they themselves are exposed to visible light. If *M. kansasii* is treated with UV radiation the majority of the cells are killed but among the survivants one has a chance to find a few that are pigmented even if grown in darkness. The progeny of these pigmented cells are also pigmented. Obviously, UV radiation caused a change in the genetic program.

In general, the distinction between phenotypic variations and genotypic variation is simple. In the former, environmental factors condition the expression of a character or group of characters simultaneously in virtually every cell in the population, while in genetic variation only a few cells in the whole population exhibit the altered character.

Historically, to determine what caused genetic variation was not a simple matter. Some assumed, for example, that a few cells in a population have the capability of changing their genetic structure in response to changes in the environment (adaptation hypothesis); others assumed that the change in the genetic structure occurs prior to the change in the environment (mutation hypothesis). In the first case, the environment directs the genetic change, whereas, in the second, the role of the environment is only to select those cells better fitted to survive. Luria and Delbrück (*64*) realized that the solution of this dilemma could only be sought by defining it in quantitative terms, which they did in the following way:

> 1. First hypothesis (mutation): There is a definite probability for any bacterium to mutate during its lifetime from "sensitive" to "resistant." Every offspring of such a mutant will be resistant, unless reverse mutation occurs. The term "resistant" means here that the bacterium will not be killed if exposed to (virus)[1], and the possibility of its interaction with (virus) is left open.
>
> 2. Second hypothesis (acquired hereditary immunity): There is a small finite probability for any bacterium to survive an attack by the (virus). Survival of an infection confers immunity not only to the individual but also to its offspring. The probability of survival in the first instance does not run in clones[2]. If we find that a bacterium survives an attack, we cannot from this information, infer that close relatives of it, other than descendants, are likely to survive the attack.
>
> The last statement contains the essential difference between the two hypotheses. On the mutation hypothesis, the mutation to resistance may occur any time prior to the addition of (virus). The culture, therefore, will contain "clones of resistant bacteria" of various sizes, whereas, the hypothesis of acquired immunity the bacteria which survive an attack by the virus will be a random sample of the culture.
>
> For the discussion of the experimental possibility of distinction between these two hypotheses, it is important to keep in mind that the offspring of a *tested* bacterium which survives is resistant on either hypothesis. Repeated tests on a bacterium at different times, and on a bacterium and its offspring, could therefore, give no information in deciding the present issue. Thus, one has to resort to less direct methods.[3]

---

[1] In their investigations, Luria and Delbrück studied the acquisition of resistance of *Escherichia coli* to bacteriophage.

[2] Clone is defined as a population of cells derived from a single bacterium.

[3] The reason for this lengthy quotation is that a clear understanding of this subject is critically important to tuberculosis therapy and to the laboratory procedures in drug sensitivity testing of the tubercle bacilli.

The method that they designed is known as the fluctuation test and is discussed later on in this chapter. This method introduced into microbiology methods of statistical analysis necessary to study large populations, a very decisive step in future developments in the biological sciences.

SURFACE PLATE COUNTS – SOLUTION OF A TECHNICAL PROBLEM

Although the tubercle bacilli were successfully grown in the laboratory as early as 1882, and other mycobacteria were cultivated in subsequent years, only in 1945 did these organisms become amenable to population analysis. In liquid media, the mycobacteria grow at the surface, forming a thick pellicle or they grow in large clumps. In 1945, Dubos (*31*) demonstrated that submerged and readily dispersed growth could be obtained in media containing synthetic nonionic surface active agents. Mostly through the efforts of Dubos, Middlebrook and their co-workers, are media available today that are suitable for population studies in the mycobacteria.

Detailed studies (*35, 36*) on the enumeration by surface plate counts of viable tubercle bacilli grown in a liquid medium containing Tween 80 (Dubos Tween-albumin medium) and made solid by the addition of agar demonstrated that:

1. The viable counts agreed well with total counts made in a Petroff-Hauser chamber.
2. Isolated bacterial cells produced colonies regularly.
3. Well-dispersed growth in Tween-albumin medium contained many isolated cells and small clumps of three to five cells.

As pointed out by Fenner (*36*), the results showed that "viable mammalian tubercle bacilli of a number of different strains can be counted by the surface drop method with much the same degree of accuracy as many other bacteria."

FLUCTUATION TEST AND ITS APPLICATION IN MYCOBACTERIOLOGY

The fluctuation test whose design is illustrated in Figure 1 consists of two experimental procedures. One is a check of the reliability of the testing procedure (left-hand side of the figure); the experiment represented at the right discriminates between the mutation and the adaptation hypotheses on the origin of genetic variation (fluctuation analysis). In the discussion of the fluctuation analysis that follows, only data obtained with the mycobacteria is given. For a more general treatment of the subject, the reader may consult available textbooks (see, for example, references *15, 43* and *82*).

The check on *the reliability of the testing procedure* is based on the principle that aliquots of a single culture must yield approximately the same number of colonies on solid medium (surface plate counts), and that any variation in the number of colonies must be due to chance alone. Likewise, if the bacteria are exposed to a selective agent on the plates, the number of colonies of resistant bacteria on different plates should be approximately the same. Theoretically, the mean number of colonies ($\bar{x}$) on the replicates should be equal to the variance (S^2). In other words, the distribution of the number of colonies on plates must conform with the Poisson series ($S^2 = \bar{x}$).

Contrary to what is generally assumed in the tuberculosis literature, the kind of experiment shown in the left hand side of Figure 1 does not give anv information about the origin of drug-resistant bacteria (see above, quotation from Luria and Delbrück). Indeed, the resistant bacteria could have originated on the solid medium by interaction with the drug (adaptive resistance) or they could have been present in the aliquots of the original culture (preadaptive resistant cells or mutants). It should be noted that Yegian and Vanderlinde (*110*), from data obtained in experiments such as this, concluded correctly that the nature of resistance of mycobacteria to streptomycin was the selection of variants that were present in the original cell population. However, the results of their experiments could as well be interpreted in terms of the adaption hypothesis as noted before (see also inducible resistance, page 101.

When *the fluctuation analysis* is applied, two kinds of results can theoretically be expected:

1. The number of colonies of drug-resistant bacteria among the various plates may be approximately the same and conform with the Poisson distribution. This result will be in agreement with the hypothesis that the resistant bacteria originate after being exposed to the drug in the solid medium.

2. The number of colonies among the plates may be markedly different (exhibit marked fluctuation). This result will be in agreement with the hypothesis that the resistant bacteria originate independently of the exposure to the drug in the solid medium.

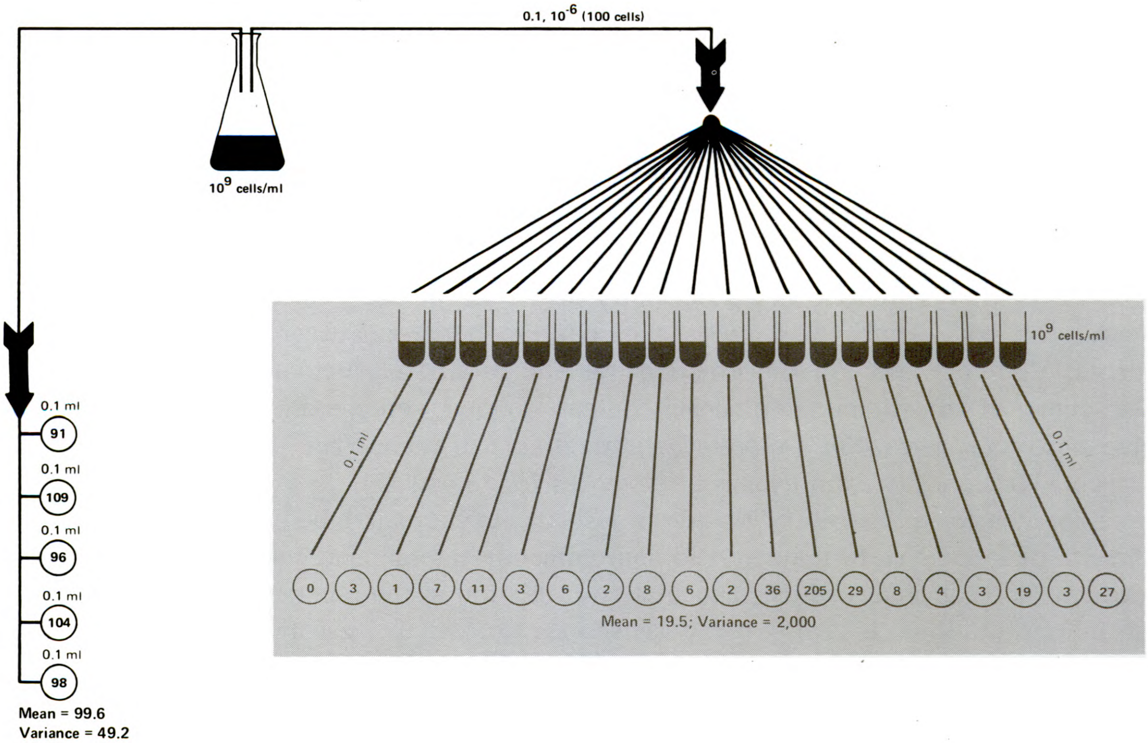


FIGURE 1. Diagrammatic representation of the fluctuation test. The figures are from an experiment in which the resistance of *M. tuberculosis* against isoniazid (0.2 μg/ml) is analyzed.

The application of the fluctuation test to a mycobacterium (*M. fortuitum, syn. M. ranae*) was first reported in 1950. Hsie and Bryson (*47*) investigated the acquisition of drug resistance to streptomycin in *M. fortuitum* growing in nutrient broth containing the tensioactive agent Triton A-20, and their results were consistent with the conclusion that resistance was due to spontaneous mutations occurring in growing populations at a frequency of about 10^{-9} mutations per bacterium per generation. The mutation frequency or mutation rate indicates the probability that a bacterium has to suffer a mutation. It is defined as the probability of a mutation per bacterium per time unit (generally expressed as a generation) and can be interpreted as the reciprocal of the average size that the bacterial population must attain for a mutation to occur. The fluctuation test was constructed in such a manner that this probability can be calculated, either from the frequency of the small identical cultures that did not yield mutants [$\mu = \frac{\ln 2}{N} \cdot \ln P_0$] (equation 1), or from the average number of mutants per culture [$r = \mu\, N_t \bullet \ln\, (N_t \bullet C \bullet \mu)$] (equation 2). In this latter equation, μ = mutation rate, P_0 = number of cultures that did not yield mutants, r = average number of mutants per culture, N_t = size of the bacterial population in the identical cultures, and C = number of small identical cultures.

In dealing with the mycobacteria, the method that requires plating the whole volume of small cultures (0.1 to 0.5 ml) onto the testing medium (drug containing plates) cannot be applied, because the long periods of incubation that are necessary result in considerable dehydration (equation 1 is applicable in this method). Luria and Delbrück's second method in which cultures of larger volume are used and aliquots of the cultures are tested (equation 2 is applicable in this method) must therefore be adopted. This equation is resolved by iteration, conveniently with the help of a computer. We have used the following program:

$$r = \mu \bullet N_t \bullet \ln\, (N_t \bullet C \bullet \mu)$$

```
100 READ R,N,C,A
110 LET X=R/(N*LOG(N*C*A))
120 PRINT "X =";X
125 LET Z=ABS(A-X)
130 LET A=X
135 IF Z<1E-14 THEN 999
140 GO TO 110
200 DATA
999 END
```

The fluctuation analysis was applied to the bacteria first by Demerec (*28*) who studied the resistance to streptomycin. In the mycobacteria, the fluctuation analysis was applied in the calculation of mutation rates in *M. fortuitum* (*9, 10*) and later on in *M. tuberculosis, M. marinum,* and in *M. kansasii* (*11, 12, 13, 14*). The available information, summarized in Table 1, indicated that mutations occur in the mycobacteria at very low, although measurable, frequencies that are comparable to those of other bacteria.

INCREASE OF THE PROPORTION OF MUTANTS WITH TIME

Luria and Delbrück (*1*) predicted that the proportion of mutants (also called mutant frequency) should increase with time, since new mutants are added to growing populations. This prediction forms the basis for another method to calculate the mutation rates. The

TABLE 1. Spontaneous Mutation Rates in the Mycobacteria

Mutation	Species	Mutation Rate	References
Sensitivity → resistance:			
Isoniazid	*M. tuberculosis*	10^{-8} - 10^{-9}	22,23
	M. avium	10^{-5} - 10^{-8}	104
	M. fortuitum	10^{-6}	94
Streptomycin	*M. tuberculosis*	10^{-8} - 10^{-9}	22
	M. avium	$1\text{-}0^{-5}$	67
	M. fortuitum	10^{-9}	47
	M. kansasii	10^{-9}	25
PAS	*M. tuberculosis*	10^{-8} - 10^{-9}	104
Ethambutol	*M. tuberculosis*	10^{-7}	22
D-cycloserine	*M. tuberculosis*	10^{-9}	24,103
Rifampin	*M. tuberculosis*	10^{-10}	109
	M. kansasii	10^{-9}	109
Conteben	*M. tuberculosis*	10^{-4} - 10^{-5}	104
Colony type:			
Transparent → opaque	*M. avium*	10^{-4} - 10^{-5}	67

procedure was used by McCarthy (*67*) experimenting with *M. avium* and by David (*22*) experimenting with *M. tuberculosis.* It consists in estimating the proportion of mutants at various intervals; a slope of a plot of the frequency of mutants against the number of generations gives the mutation rate. This slope is described by the following equation: $\mu = 2 \cdot \ln 2 \cdot \left(\frac{M_t}{N_t} - \frac{M_o}{N_o}\right) / g$ (equation 4) in which M_o / N_o = proportion of mutants at time zero, M_t / N_t = proportion of mutants at time t, and g is the number of generations that elapsed between times t_o and t [(g $= \frac{\log N_t - \log N_o}{0.031}$) (equation 3)].

When the prediction of Luria and Delbrück is pursued, a hypothetical situation can be conceived in which mutants continue to accumulate so that they will eventually predominate. The time required to change the proportion of mutants to some determined proportion could be calculated from equation 5: $t_1 = - 1/\mu \ln \frac{N_t}{N_o}$ where μ = mutation rate, N_t = proportion of mutants at time t_1, and N_o = proportion of mutants at time t_o (*62*).

Thus, for a mutation rate of about 2.0×10^{-8}, the time required to obtain 50% of Inh^r mutants from a population without any mutants initially may be $t_1 = - \frac{1}{2\times10^{-8}} \ln \frac{0.5}{0.1}$ or about 98,000 years (assuming one generation per day). Actually, with the observed low rates of mutation, the genetic constitution of the bacterial population remains stable for as long as selective pressures do not contribute to increase the frequency of mutants.

Hayes (*43*) listed two reasons for this. First, as the number of mutants increases, the number of the wild-type bacteria decreases and therefore fewer and fewer mutations occur. On the other hand, as the number of mutants increases, the probability of *back mutation* to the wild type also increases. Thus, a state of equilibrium is established.

Selective Mechanisms

INTRODUCTION

A discussion of selective mechanisms in populations of the mycobacteria is almost limited to the phenomenon of drug resustance. However, we will briefly discuss the general principles of selection.

Atwood, Schneider, and Ryan (*1*) defined selection as "those factors other than mutation which influence the frequencies of mutants in populations." A change in the genetic constitution of a population (or the relative frequencies of the genotypes present) is caused by the introduction in the system of a factor or factors that favor the survival of one genotype in relation to another. These factors may be a consequence of the mutation itself (differences between parent and mutant in growth rates, sensitivity to bacterial metabolites, viability), or may be externally imposed upon the population.

The smooth-rough variation in *Brucella abortus* investigated by Braun and his associates (*15*) is an illustrative example of a selective force that evolves in a closed system. The parent, smooth *B. abortus* in medium containing D-asparagine, synthesized D-alanine which accumulates in the medium. The smooth cells are more susceptible to D-alanine than the rough variants are, and thus a situation develops in which the rough mutants become progressively established in the population. In medium containing L-asparagine, valine accumulates instead of D-alanine, and in this case, valine resistant mucoid variants become the predominant genotype. Other examples may be found in Braun's book.

Fregnan, Randall, and Smith (*37*) described a similar phenomenon in a scotochromogenic mycobacterium. During their investigations on the mycoside content of the mycobacteria they observed that in prolonged incubation on medium containing D-malic acid, the smooth parent organisms were progressively substituted for by a rough variant. At time zero, the frequency of the rough variants of about 10^{-3} increased to about 0.5 after 4 months of incubation. Although the phenomenon was not further examined, it is possible that changes in the evolving environment (whether decreased oxygen tension, pH changes, accumulation of metabolic products, or other) favored the establishment of the mutant genotype. Other examples of selection of genetic variants are loss of virulence of pathogenic mycobacteria entertained in the laboratory for long periods of time, which may be recovered through passages in animals and the observation that *M. avium* recovered from the tissues of rabbits retain the antigenic structure of the strain used for injection, whereas those isolated from chickens often exhibit different antigenic structure (*15*). These and many other examples of a similar nature among the mycobacteria unfortunately have not been analyzed by the methods of population genetics.

For reasons already listed, the genetic constitution of a population remains stable through many transfers in the laboratory if large inocula are used, as is generally the case. If single cell transfers are done, however, one is bound to obtain a population whose genetic structure may differ markedly from the original one. The probability of selecting a particular variant depends upon the mutation frequency and is, of course, very low. It can be increased by imposing a selective pressure directed at eliminating the wild-type parental cells, and this can be accomplished in two distinct ways. In one, the mutant is selected without ever exposing the population to a selective agent, and it is called *indirect selection of mutants;* in the other, selection is achieved by exposing the population to an agent that inhibits the growth of the parents without interfering with the growth of the mutant and it is called *direct selection of mutants.*

INDIRECT SELECTION OF MUTANTS

In this section, the Cavalli-Sforza and Lederberg (*20*) method of isolating preadaptive mutants by the selection of their siblings is discussed. The Lederberg and Lederberg replica plating method (*60*) is another method for indirect selection of mutants.

The sib-selection of mutants is an extension of Luria and Delbrück's fluctuation test. In a fluctuation test, the culture in which a mutation occurred the earliest yields the largest proportion of mutants. Therefore, successive fluctuation tests with the culture that yielded the largest mutant clone in the previous one should result in a stepwise enrichment of the population in mutants. Cavalli-Sforza and Lederberg, who conceived this experiment, applied it in the indirect selection of streptomycin and chloramphenicol resistant mutants of *Staphylococcus aureus* and *Escherichia coli* and succeeded in enriching the populations in mutants. Since at no time were the bacteria exposed to the drugs, the experiment proved beyond any doubt the spontaneous nature of mutation.

The procedure was applied in an investigation of resistance to isoniazid in the tubercle bacilli (*23*). In these experiments, the proportion of Inh^r mutants was increased 25 times in four cycles of selection. Since each selective cycle took 1 month, the experiments were not pursued further on the assumption that the 25-fold increase was significant evidence that resistance to isoniazid was due to spontaneous mutation.

DIRECT SELECTION OF MUTANTS

Since the mutation rate is the reciprocal of the average size the bacterial population must reach for a mutation to occur, the greatest chance of finding a mutant is when the size of the population reaches that figure. If a bactericidal drug is added to a growing population at a time when the cell concentration is much lower, most of the cells are killed and the few remaining survivors are sensitive bacteria that escaped the killing effect of the drug (persistors). These survivors do not divide for as long as active drug remains in the medium. On the other hand, if the drug is added at a time when the cell concentration is high, the chances are that a few mutants will be present, and therefore, growth becomes apparent even if active drug remains in the medium. From the precedent it is clear that a population of drug-resistant mutants cannot be obtained unless a mutant is present at the time the selective agent is added.

The foregoing allows the analysis of direct selection. The various factors that determine the shift in the frequency of mutants are as follows:

1. The rate of killing of the wild-type parents.
2. The size of the mutant clone at the time the selective agent is added.
3. The growth rate of mutants.
4. Environmental changes that occurred prior to or during the action of the selective agent.

The various chemotherapeutic agents exert their killing effect only on dividing cells, and therefore, a quantitative analysis of the rate of killing necessitates the use of actively growing cultures.

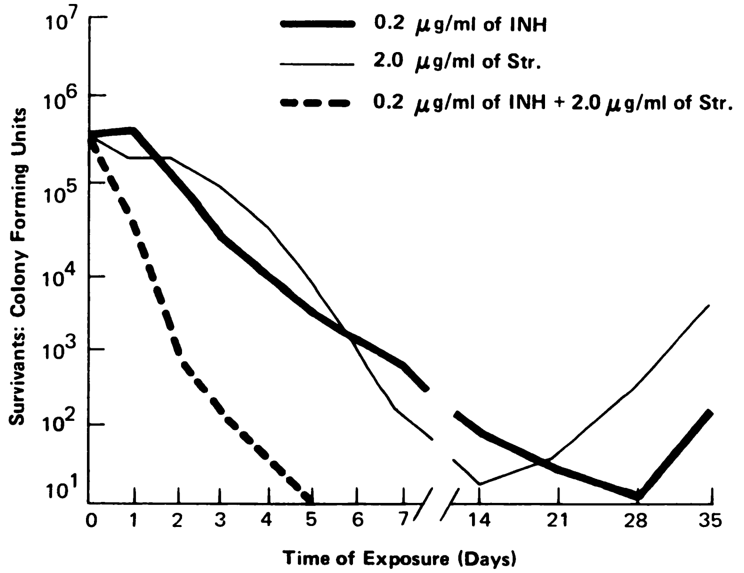
Experiments with the tubercle bacilli showed that the product of the drug concentration, by the time of exposure necessary to inhibit bacterial growth, was a constant (*2, 3, 7, 9*). This relationship was obtained in the tubercle bacilli in relation to isoniazid, streptomycin, ethambutol, and rifampin. Whether they held true in the interaction of other drugs and the tubercle bacilli, or other mycobacteria, has not yet been ascertained. The rate of killing the parent organism may be determined from the slope of the curve obtained by plotting the logarithm of the fraction of survivors against the time of exposure.

In investigation of drug resistance in the mycobacteria, the majority of the investigators have used one or more of the following experimental designs.

A. Figure 2 summarizes an experiment in which the drugs were added to a growing culture. Aliquots were taken at regular intervals, and the survival was determined by surface plate counts. The bacterial population decreased regularly for about 14 days (streptomycin) and 28 days (isoniazid), and then the cell population began to increase. When the population was tested for drug resistance during the increase, it was found to be resistant.

B. The experiment shown in Figure 3 is similar to the previous one, except that at each time a sample from the culture was plated on both drug-free and drug-containing medium. In the first experiment, there was no way of knowing what happened before the increase began, whereas the data from the second experiment shows that the point of inflexion of the curve corresponded to the moment in time when the size of the resistant clone was equal to the number of survivors.

C. A third type of experiment is shown in Figure 4. In this experiment, a culture was incubated to the stationary phase, samples were taken, and the survivors at increasing concentrations of isoniazid were determined by surface plate counts on a



(modified from Mitchison, 68)

FIGURE 2. The bacterial effect of isoniazid and streptomycin, alone and combined.

solid medium containing the indicated concentration of the drug. The survival curve shows a very distinct discontinuity of about 0.005 μg/ml of Inh. The ratio of the number of survivors to the total number of cells in the population can be taken to approximate the mutation rate,[4] provided that no selection occurred before and that resistance in the particular case under study is caused by spontaneous mutation. At the concentration of 0.2 μg/ml this ratio is about 10^{-7}.

D. The successive transfer of bacteria in media with increasing concentrations of a drug constitutes another, and very extensively used, procedure for the direct selection of drug-resistant mutants. In the usual way, the bacteria are inoculated in a series of tubes containing medium with increasing concentrations of the drug. The cultures are incubated for a suitable time, and from the culture containing the highest concentration of the drug in which growth is apparent, a transfer is made to another similar series. The process is repeated until a population growing at a high concentration of drug is obtained. Because of the mechanics of the experiment, the emergence of resistance appears to be due to training of the bacteria. What actually happens is that the various transfers at low concentrations increase the probability of a mutation occuring and once this happens, the successive transfers become equivalent to cycles of enrichment by direct selection. The time it takes for the growth to be visible at each concentration becomes shorter in successive cycles of enrichments because the time lag for a culture to give visible growth is a function of the size of the inoculum (*111*).

The four kinds of experiments described, all indicate that the mutants become established progressively in a population subject to a selective pressure. The various examples were of direct selection, since a drug was used as the selective agent. At this point, there are a number of questions that may be asked: for example, how the point of inflection in the curve in Figures 2 and 3 is influenced by the original concentration of mutants, by their growth rate, by products released from the dead cells that may compete with the action of the drug, and by the limitation of growth due to depletion of nutrients in the medium.

It was noted earlier that the growth of the mycobacteria in stationary or batch cultures is exponential for a short period that is followed by a prolonged arithmetic growth phase. During this phase there is a constant decrease in growth rate supposedly in consequence of a growth limiting condition that develops in the culture. The continuous changes in the environmental and cell properties that occur during growth makes it difficult to analyze accurately the rate of genetic variation and the mechanisms of selection in bacterial populations. These difficulties are obviated in continuous culture devices, in which the nutrients are supplied at a constant rate to the culture. Under these conditions, the culture attains a "steady state" in which the total number of cells and the growth rate remain constant. In spite of their considerable importance, continuous culture devices have not been applied to mycobacteriology.

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[4] The mutation rate is the reciprocal of the average size the population has to reach for a mutation to occur.

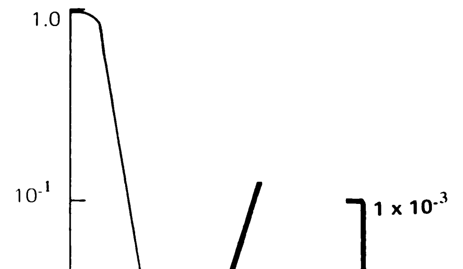

FIGURE 3. Direct selection of isoniazid-resistant mutants of the tubercle bacilli (adapted from David and Newman, *23*).

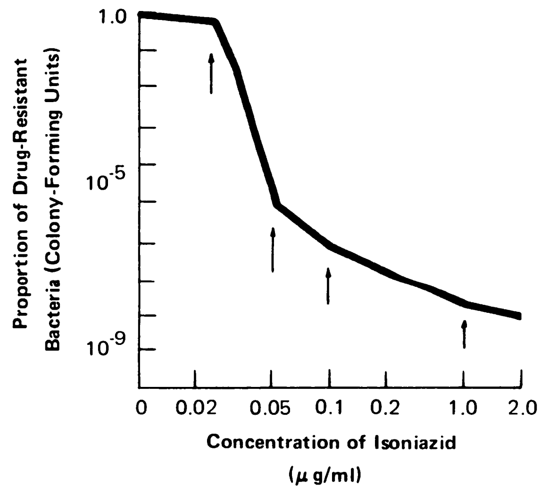

FIGURE 4. Proportion of drug-resistant bacteria in a population of the tubercle bacilli, strain $H_{37}Rv$, at increasing concentrations of isoniazid. Note the sharp discontinuities (↑) (adapted from Tsukamura, *104*).

Molecular Basis of Mutation

INTRODUCTION

The diagram in Figure 5 illustrates the mechanism of protein synthesis. The following are the important features:

1. The order in which the nucleotide bases (adenine, guanine, cytosine and thymine) are arranged in the DNA molecules constitute a *code.*

2. Each of the 20 amino acids commonly found in the proteins is represented by at least one sequence of three bases (a triplet).

3. The sequence of the bases in the DNA determines the sequence of the amino acids in polypeptides.

4. The first step in protein synthesis is the transcription of the code into a complementary strand of RNA (called *m*RNA, messenger RNA). (In *m*RNA uracil substitutes for thymine.) The triplet bases in *m*RNA that are the code for one amino acid are called a *codon.* For example, UUU is the *codon* for phenylalanine.

5. The information transcribed in the *m*RNA is then translated into the amino acid sequence of the polypeptide in the ribosomes.

6. For each of the amino acids there is at least another kind of RNA (*t*RNA, transfer RNA). The amino acid reacts with the *t*RNA to form the aminoacyl-RNA. The *t*RNA contains a triplet, called the *anti-codon,* which is complementary to the *codon.* For example, the *anti-codon* for UUU is AAA.

7. As the *m* RNA moves along the ribosomes, the aminoacyl-RNA's are added one by one as determined by the complementarity of the *codon* and the *anti-codon* (translation).

The genetic code has now been deciphered. Note that more than one triplet codes a single amino acid. This is called *degeneracy.* Certain triplets (UGA, UAG, UAA) do not correspond to any amino acid. These are called *nonsense codons,* and they seem to serve as punctuation signals to indicate the termination of a gene coding a certain protein. At least one starting point of protein synthesis is known, and it relates to the occurrence in *m*RNA of the *codon* for N-formyl methionine (GUG) and of a specific N-formyl methionine (GUG) and of a specific N-formyl methionine-tRNA. The initiation point assures the proper reading of the *codon;* since each amino acid is represented by a triplet, it is essential that it is translated from the first nucleotide base. Otherwise, another amino acid would be incorporated into the growing polypeptide chain (*reading frame* shift).

Mutations are the result of alteration in the structure of the DNA molecule. The kinds of changes in DNA serve as the basis for classifying mutations. Thus, one may have transitions (a purine base is substituted by another, or a pyrimidine base is substituted by another), transversions (when a purine base is substituted by a pyrimidine base, or vice versa) and additions or deletions of nucleotide bases.

The deletion of a large portion of DNA results in complete loss of the ability to form a protein; the DNA cannot, of course, be restored. When a single base is deleted or a substitution occurs (point mutation), the DNA can be restored, either by genetic recombination (deletions) or further mutations (substitution). Mutations may also be classified on the basis of the product (protein). Thus, a frame-shift mutation is usually caused by deletions or insertions, whereas base substitutions result in missence mutations (wrong amino acid) or in nonsense mutations (no amino acid added, chain termination).

Not all mutations lead necessarily to nonfunctional proteins and may, therefore, remain undetected. On the other hand, since the code is degenerate, some mutations will not affect the amino acid. For example, suppose adenine is substituted for guanine in the triplet UCG, that codes serine. The resulting triplet UCA also codes serine, and therefore, the amino acid will appear in the protein at the right location. These mutations are called *silent mutations.*

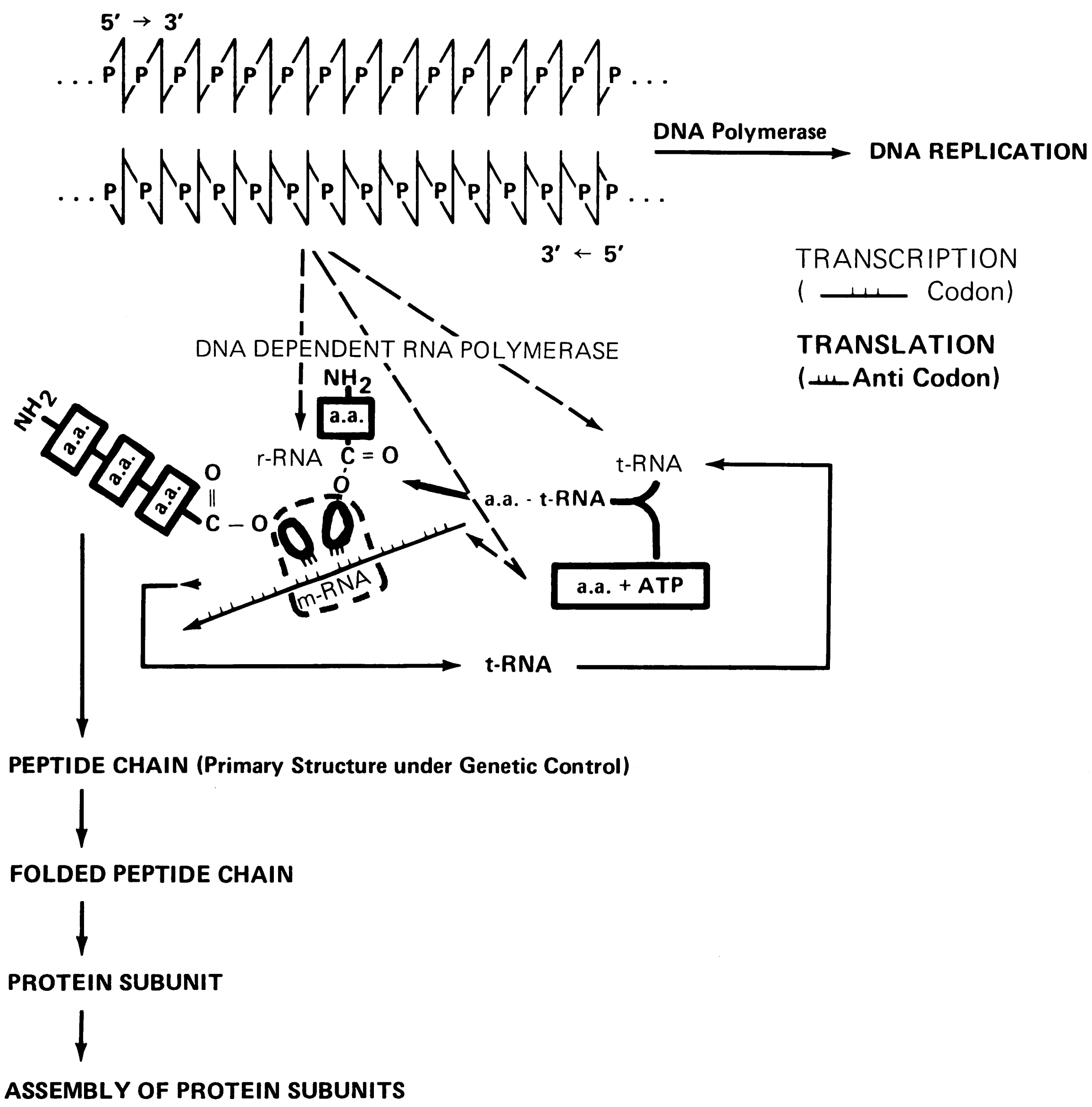

FIGURE 5. Schematic representation of DNA, RNA, and protein synthesis.

Spontaneous mutations are probably due to random errors during DNA replication. The frequency of copy errors can be greatly enhanced by a variety of agents (mutagens). The study of the mechanism of action of these mutagens has provided information on the molecular basis of mutations.

EFFECT OF MUTAGENS ON THE MYCOBACTERIA

•Ultraviolet Radiation

If a suspension of bacteria is irradiated with ultraviolet (UV) light of a wavelength of 2537 Å (germicidal lamp) and surface plate counts are taken at regular intervals, the surviving fraction can be plotted against the time of exposure or dose. The resulting plot is called a UV survival curve. Figure 6 shows UV survival curves of various mycobacteria. The reader will notice that, at the lower doses of radiation, the curve bends smoothly downward and that it becomes linear when the dose reaches a certain threshold, Since the plot is logarithmic, the linear portion of the curve indicates that the killing effect was exponential.

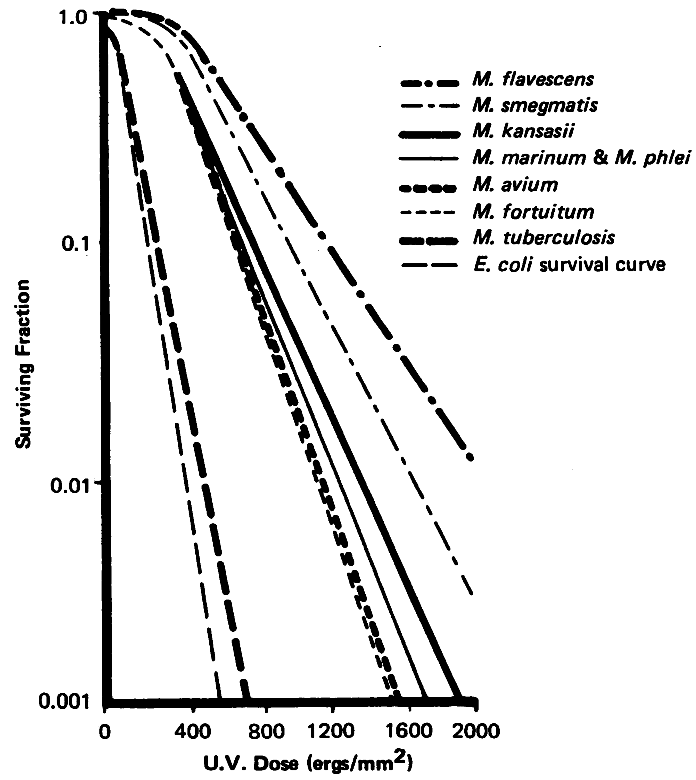

FIGURE 6. Sensitivity of mycobacteria to UV light (*M. tuberculosis, M. fortuitum, M. avium, M. marinum, M. phlei, M. kansasii, M. smegmatis* and *M. flavescens).* The survival curve of *E. coli* is also shown for comparative purposes (adapted from David, *25*).

The initial shoulder is characteristic of many biological systems, and its current interpretation is as follows: The UV radiation alters the DNA bases which leads to the formation of dimers. DNA is damaged as soon as it is exposed to radiation, and the processes of enzymatic repair that follow are slow. As the dose of radiation increases the number of DNA lesions that are repaired decreases and, therefore, the plot obtained has the shape of a shoulder. In other words, at low doses of radiation the survival [S] = number of lesions in the DNA – number of repaired lesions. When the dose of radiation is higher the repair mechanisms are saturated, and the inactivation becomes an exponential function of the dose (linear portion of the curve).

Suppose that concurrently with the surface plate counts as described above, one also would do platings on a medium containing a drug. In an experiment such as this, frequency of mutants resistant to the indicated drugs increases with the dose of radiation (*108*). The drug-resistant mutants are the easiest kind to isolate. Since virtually every property of the bacteria can change by mutation, the appropriate conditions must be developed to isolate the kind of mutant desired. Thus, mutants of *M. phlei* that require exogenous amino acids or nucleotides can be induced by irradiating the culture with UV radiation at doses that give 5% or lower survival. One way to select this kind of mutant, called an auxotrophic mutant, is to plate the irradiated culture on medium supplemented with the exogenous metabolities. The growth from this medium can then be transferred by replica plating to medium lacking the growth factor. These mutants that require specific nutrients are also called *nutritional mutants,* and the corresponding mutations are *conditional lethal mutations* because the survival of the mutant depends on whether the specific nutrient is exogenously available or not.

The UV radiation produces photochemical changes in the DNA, the most important of which are hydration and dimer formation in the pyrimidine bases. For the biological inactivation of DNA, thymine dimer formation is the more important lesion. Although the mechanism of mutation is still unclear, it is possible that the abnormal replication of the segment containing the dimer is what causes the mutation.

We have already noted that the UV-damaged DNA can be repaired. The two most important repair mechanisms are *photoreactivation* and *dark recovery.*

Photoreactivation refers to the recovery of lethally damaged cells by simultaneous or postirradiation of cells by light (*56a*). Action spectra of photoreactivation have shown that the most effective radiation is in the range 3000 to 5000 Å. The mechanism of photoreactivation is the enzymatic splitting of the pyrimidine dimers, chiefly thymine.

Dark recovery refers to the recovery of irradiated cells that are held in liquid for several hours before plating (*79*). This is also called liquid holding recovery. The mechanism of dark recovery is the enzymatic excision of the damaged segment of DNA and subsequent repair synthesis (*73*).

The excision-repair enzymes may play an important role in correcting copy errors during DNA replication, therefore reducing the frequency of spontaneous mutations. Thus, strains of bacteria that exhibit unusually high mutation rates (mutator strains) are usually very sensitive to UV radiation.

The information available on repair mechanisms in the mycobacteria is very fragmentary. Photoreactivation was demonstrated in several mycobacteria (*23a, 25, 88a*) and dark repair was demonstrated in *M. avium* (*25, 67a, 88a*).

The molecular basis of these phenomena has not been investigated in the mycobacteria. It is of interest to note, however, that the kinetic data available on the mycobacteria do not differ significantly from the published data on other bacteria.

•Chemical Mutagenesis

Copy errors can be greatly increased by nucleotide base analogs or by substances that react chemically with the nucleotide bases. Nitrosoguanidine, hydroxylamine, and ethyl methanesulfonate were successfully used to induce auxotrophic mutants in *M. phlei* and *M. smegmatis* (*58, 59, 93*).

Genetic Transfer in the Mycobacteria

Three mechanisms of transfer of genetic material from a bacterial cell (donor cell) to another (recipient cell) are known: conjugation, transformation, and transduction. Transduction (*40, 52, 53, 58, 59, 84, 92*) and conjugation (*70, 102*) in the mycobacteria were reported. Since there is very little information on the subject as far as the mycobacteria are concerned, it will not be discussed.

Mycobacteriophages

INTRODUCTION

Bacterial viruses (bacteriophages) are very widespread in nature and are found as parasites in almost every taxonomic group. Usually, these viruses parasitize bacteria within well defined taxonomic boundaries, and this specificity towards a certain group defines their host range. The viral particles (virion) may be of different sizes and shapes. Some are complex structures composed of a head, a tail and an end plate at the tip of the tail that serves as an organ of attachment to the host bacteria, like the bacteriophage T_4 (Figure 7); others, like the bacteriophage M_{13}, are filamentous; whereas still others may be polyhedric like the bacteriophage ϕ x 174. Basically, the virion consists of a protein shell (called the capsid) which encloses a nucleic acid. Again, there is a variety of possibilities among these viruses: in some the nucleic acid is double-strand DNA, in others the DNA is single-strand, and in others the nucleic acid is RNA.

To grow, the bacteriophages must find a suitable bacterial host. The various phases of their growth may be summarized as follows:

1. The virion attaches to the cell surface.
2. The viral nucleic acid is injected into the cell, the protein remaining on the outside.
3. The viral nucleic acid replicates.
4. The viral nucleic acid directs the synthesis of the structural proteins and other essential proteins.
5. The nucleic acid and the proteins assemble to form new virus particles.
6. The complete virion is released from the cell usually, but not necessarily, by lysis.

When a bacteriophage infects the host, the two major outcomes are:

1. Either the death of the host by lysis at the end of the replicative cycle; or
2. A permanent association between the virus and the host, as follows:
    a. The DNA of the viruses may integrate in the host's DNA, and thus the bacteriophage becomes part of the genetic constitution of a bacterium. This is called lysogeny, and the virus is said to be in the prophage state.
    b. The bacteriophages remain associated to a bacterial culture for long periods of time, but still temporarily. This is called pseudolysogeny or the carrier state.
    c. The viruses are released from the host cells without cell lysis, and the bacteria continue to support virus growth for long periods of time. This phenonemon does not have a specific designation, and is found associated with the *E. coli* bacteriophages fd and M_{13} (*46, 83*).

The bacteriophages that always give a lytic response are called virulent; those whose DNA may take the form of a prophage are called temperate. Clearly, only the temperate bacteriophages can give a lysogenic response. In the carrier state condition a fraction of the bacterial population becomes refractory to viral infection, and as this condition is progressively established, the virus replicates and lyses the susceptible fraction of the population. While in the lysogenic state, the prophage is part of the bacterial chromosome, and, therefore, the progeny of a lysogenic cell is also lysogenic. In the pseudolysogeny or carrier state, the infected bacterium does not have a progeny because it must lyse. In other words, pseudolysogeny is associated with a population of bacteria, whereas lysogeny is associated with each individual within a population.

The bacteriophages whose hosts are the mycobacteria are called mycobacteriophages. The first report on mycobacteriophages was by Gardner and Weiser (*39*). Table 2 is a partial list of mycobacteriophages and their hosts. The properties of these viruses will be reviewed.

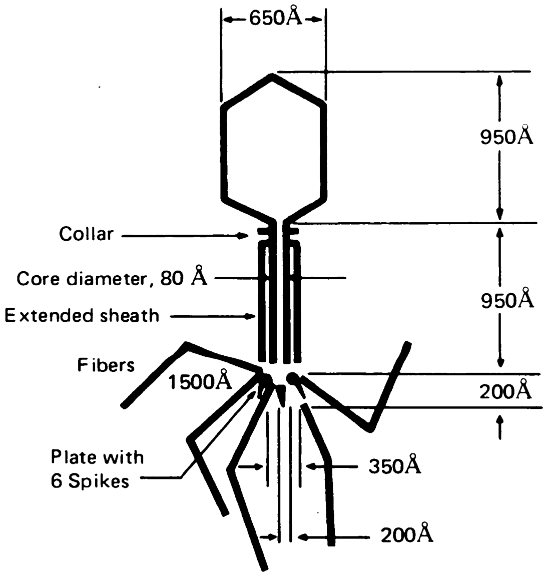

FIGURE 7. Structure of the T-even bacteriophage particle (adapted from Stent, *90*).

TABLE 2. Partial List of Mycobacteriophages and Their Host Bacteria

Mycobacteriophage	Indicator Host	Reference
DS6A	*M. tuberculosis,* strain $H_{37}Rv$	76
BK-1	*M. tuberculosis,* $H_{37}Rv$	4
BK-3	*M. smegmatis*	5
BK-4	*M. fortuitum*	5
D_{29}	*M. smegmatis* 607	38
Bo 1	*M. smegmatis*	54
Bo 2	*M. phlei*	54
Bo 3	*M. phlei*	54
Bo 4	*M. fortuitum*	54
Bo 5	*M. vaccae*	54
BO 6	*M. fortuitum*	54

PHYSICAL AND CHEMICAL STRUCTURE

All the mycobacteriophages so far viewed under the electron microscope are complex viruses (Figure 8). Thus, they consist of a head and a tail. Other structures shown in Figure 1 were not clearly demonstrated in the mycobacteriophages. Particularly, the organization of the tip of the tail is unclear.

The capsid is made of protein, but it may also contain structural lipids in some of the mycobacteriophages. The evidence for the occurrence of structural lipids is as follows:

1. Most mycobacteriophages are inactivated by organic solvents (*13, 50, 74, 88*) (Figure 9).
2. Observed with the electron microscope, the structure of mycobacteriophage D_{29} treated with ether, chloroform, or methanol is markedly damaged. Many heads are empty and bacteriophage debris is observed (*88*).
3. Lipid substances were extracted with chloroform-methanol from purified preparations of mycobacteriophages D_{29}, R, and DS6A (*13, 14, 34*).

The nature of these structural lipids is undecided. Thus, those extracted from mycobacteriophage DS6A were said to be phospholipids (*14*), but those from mycobacteriophage D_{29} did not seem to be phospholipids and remained unidentified (*50*).

Structural lipids are not common among the bacteriophages. Besides the above-mentioned mycobacteriophages, the only other lipid-containing bacteriophage reported was PM_2 whose host is a marine *Pseudomonas.* This is a polyhedric particle, apparently surrounded by an envelope. The lipids of this phage were identified as phospholipids. According to Braunstein and Franklin (*16*), the lipid content of the virion is significantly different from that of the host. This suggested that the phage lipid is not taken from the membrane by a process of budding, but that the virus somehow alters the synthesis of lipids in the host.

As far as the nature of the nucleic acids is concerned, all mycobacteriophages analyzed from this point of view contained double-strand DNA (*80, 88, 100*).

DETECTION AND QUANTITATION

Ordinarily, the titer of viral particles in a suspension is estimated by adding aliquots of serial dilutions of the original suspension to a suspension of the host bacteria (indicator strain). The suspension is mixed thoroughly and spread evenly over the surface of an agar

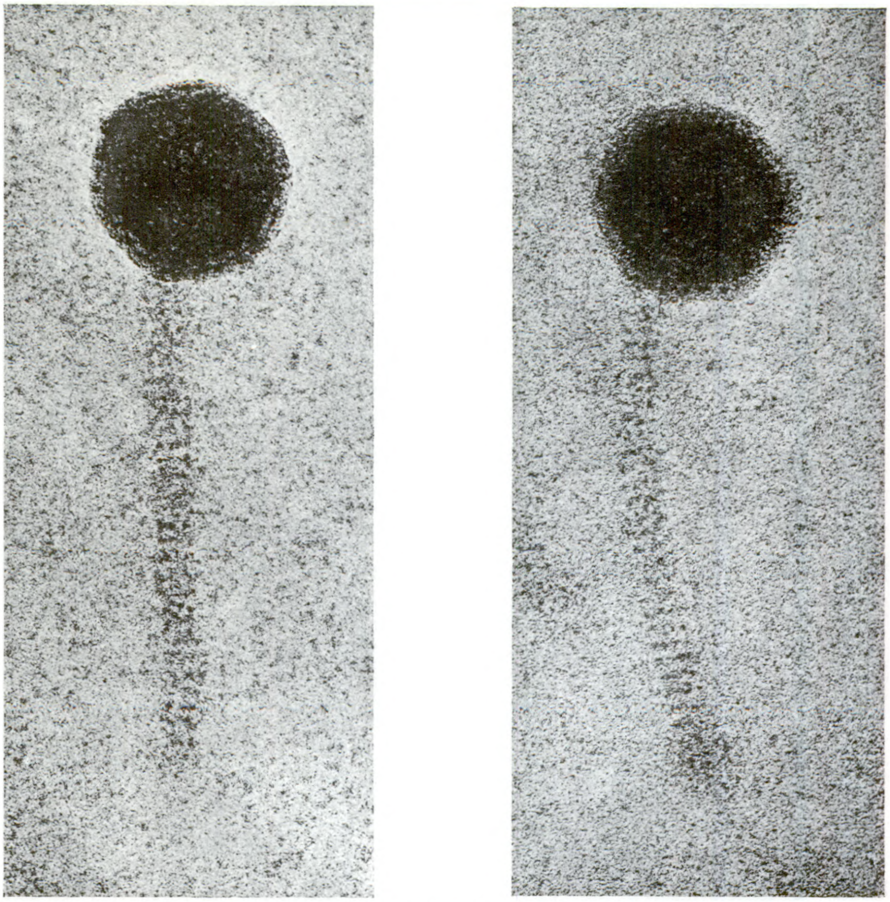

FIGURE 8. Mycobacteriophages Bk-1 and D_{29} (adapted from Kölbel and Mohelska, *57a*).

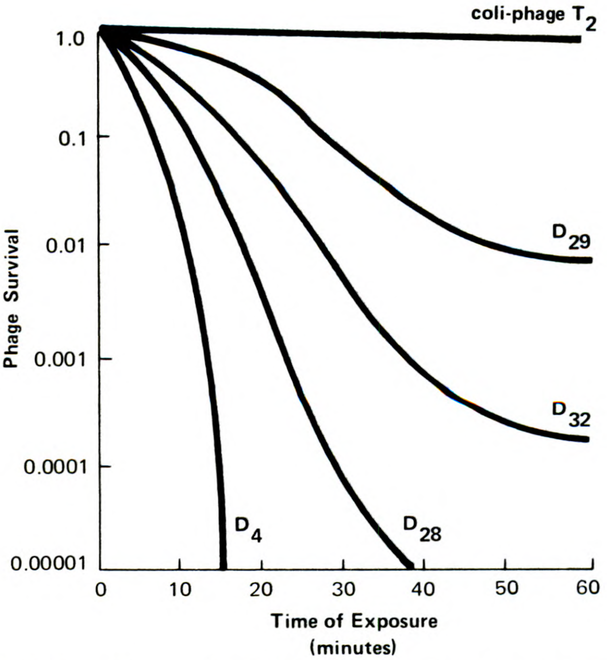

FIGURE 9. Survival of Coli-phage T_2 and the indicated mycobacteriophages as a function of the time of exposure to ether (adapted from Sellers and Tokunaga, *88*).

base medium in Petri dishes. The bacterial suspension may be in a small volume of broth or in a nutrient soft agar (overlay technique). The latter is more accurate and is thus the preferred technique.

In the overlay, the bacteriophage infects one cell which will eventually lyse and release the viral progeny. These, in turn, infect the surrounding bacteria and the process repeats itself. Under the proper conditions, the non-infected cells produce confluent growth in the overlay while no growth is observed at places where a bacterium was infected. These areas of no growth are called plaques. The plaques are usually circular, and may be clear and well delineated or may be turbid and not so well delineated. When the dilution of the phage suspension that yielded a certain number of plaques is known, the number of plaque forming units (P.F.U.) present in the original suspension can be determined. Because some of the viral particles in the suspension may be of inactivated virus, the term P.F.U. is used rather than the actual number of particles. The actual number of particles is estimated by other methods, for example, counting under the electron microscope.

If detection of the virus is the only purpose, the suspension can be spotted onto a bacterial lawn on solid medium or added to a culture in liquid medium. Clearing that can be maintained by serial transfers is indicative of the presence of a bacteriophage. Indeed a lytic agent that cannot reproduce itself would be diluted out after a few transfers.

ADSORPTION

Suppose that one has a virus suspension of known titer and a suspension of its bacterial host, and that the following experiment is done:

1. Infect the culture with the virus so that the ratio of viruses to bacteria is about 1.0. Mix well.
2. Immediately after infection, and then at regular intervals, take a sample and dilute to 1/100.
3. Centrifuge the diluted samples at about 3,000 rpm to remove the bacteria.
4. Titrate the viruses in the supernatants.

The results of experiments such as this are shown in Figure 10. The figure was constructed by plotting on semi-logarithmic paper the fraction of phages in the supernatant (titer of phages at time t / initial titer of phages, P/Po) against time; it shows a decrease in phage titer that is exponential in respect to time. If, on the other hand, the cell sediment is diluted and mixed with the indicator strain in an overlay, plaques are also formed. In other words, the fraction of viruses that disappeared from the supernatants is found in the cell sediment. In the cell sediment observed with the electron microscope, the viruses are shown fixed to the cell surface by the tip of their tails. This fixation of the bacteriophages to the cell surface is called *adsorption.*

In the first of the above experiments, the free bacteriophages (supernatant) were titrated, whereas in the second experiment the bacteria that adsorbed the bacteriophages (sediment) were titrated. Whether it is a single virus particle or an infected bacterium, the unit that forms a single plaque is called *infective center.*

Most phages do not adsorb in distilled water. The composition of the medium, particularly its ionic composition and pH, and the temperature play an important role. Thus, for example, the rate of adsorption of mycobacteriophages D_{28} and D_{29} is increased by supplementing the medium with calcium (*11*). Although the conditions of adsorption of the mycobacteriophages were not investigated in great detail, the above observation constitutes the basis for the addition of calcium to media used in mycobacteriophage work (*77*). It should be remembered that the optimal conditions of maximal adsorption vary with the phage. Another important observation is that Tween 80, ordinarily added to liquid media to obtain dispersed growth of the mycobacteria, prevents adsorption; however, Tween 80 does not inactivate the mycobacteriophage (*11, 86*).

The time course of adsorption represented in Figure 10A shows that adsorption follows the kinetics of first-order reactions as shown in equation 6.

$$\frac{dp}{dt} = -K.P.N.$$

in which P is the concentration of unadsorbed bacteriophages after a time t, N is the concentration of cells in the original mixture, and K is a constant (adsorption rate constant).

By integration,

$$\frac{P}{P_o} = e^{-K.N.t.} \text{ (equation 7)}$$

in which e is the base of the Neperian logarithms, Po is the initial concentration of bacteriophages, and the other symbols have the same meaning as above.

When the equation is converted, equation 8 is obtained:

$$\ln \frac{P}{Po} = -K.N.t.$$

or in logarithms of base 10, equation 9 is obtained:

$$\log_{10} \frac{P}{Po} = \frac{-1}{2.3} K.N.t.$$

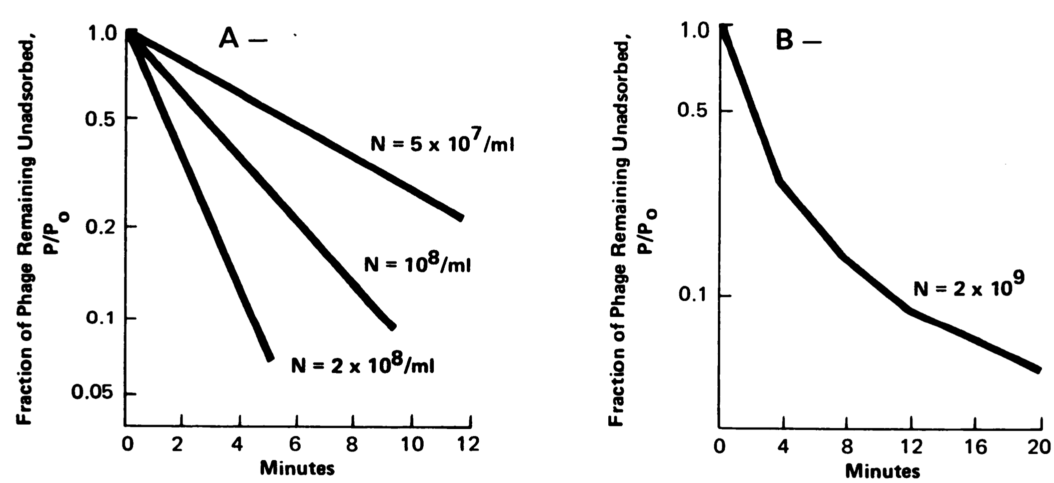

FIGURE 10. Kinetics of adsorption of bacteriophage T_4 to suspension of *E. coli* at various cell densities (A), and kinetics of adsorption of mycobacteriophage D_{29} to *M. fortuitum* (B). (Curve A is adapted from Stent, *90*, and curve B from Bowman, *11*. Note the differences in the time scale.)

(The Neperian logarithms are converted into logarithms of base 10 as follows:

$\mathrm{ln}x = \log_{10} x/\log_{10} e$; since $e = 2.718$, then $\mathrm{ln}x = \log_{10}$

$x/o.4345 = 2.3 \log_{10} x$, or $\ln x = \ln_{10} \log_{10} x$).

These equations show: 1) that the time course of bacteriophage adsorption follows the kinetics of first-order reactions in respect to both the concentration of phage and the concentration of bacteria; 2) that the rate of adsorption increases with the concentration of bacteria; and 3) that the logarithm of the fraction of initial phage remaining unadsorbed decreases linearly with time at a rate that is proportional to the concentration of the bacteria in the mixture.

Bowman's (*11*) kinetic study on the adsorption of mycobacteriphages is the only one available in the literature; Figure 10B is taken from this work. Quite obviously the adsorptive kinetics shown deviates from the above description. The figure shows that the rate of adsorption of unadsorbed phage decreases with continued incubation. What factors determine this anomalous behavior are not known.

Investigations on the chemical nature of the cell's receptors showed that the mycobacteriophages are inactivated by substances extracted from the cells with organic solvents (*17, 48, 101*). The receptors described were lipopoly-saccharides or phospholipids. The inactivation of mycobacteriophage *phlei* by an aqueous extract of *M. phlei* was also reported (*18*).

GROWTH CYCLE

"The multiplication of the bacteriophages is extremely rapid, and, notably, it is by successive bursts, each increment being separated by an interval of 75 minutes," thus d'Herelle (*21*) described the growth of the bacteriophages. To separate one cycle from the next was a major difficulty in the study of phage multiplication until Ellis and Delbrück, in 1940 (*33*), designed the *one-step* growth experiment (Figure 11) that is now a classic and one of the most important experiments in virology.

The basic principle in the experiment is to dilute the infected cells shortly after adsorption, which stops further adsorption of the input phage and prevents the progeny phage from starting a second cycle of multiplication. If platings for infective centers are made at regular intervals after adsorption, the titer remains constant for a time, characteristic of the virus-host system (latent period), and then it increases rapidly (raise period) before it levels off. The latent period is the period of intracellular growth of the bacteriophage. To find out what happens to the viral DNA, during the latent period the cells must be artificially broken. This was first done by Doermann (*30*) who demonstrated that during the latent period there is a time when no infective phage particles are found. The period of disappearance of infectivity is called the *eclipse period* of vegetative replication; it terminates when the protein and nucleic acid components of the virus assemble (maturation).

The following parameters of virus growth that are given by the above experiments should be obtained in characterizing any bacteriophage-host interaction:

1. The initial number of viruses (Po).
2. The initial number of bacteria (Bo).
3. The number of phages adsorbed, or the multiplicity of infection (m.o.i.).

4. The eclipse period.
5. The latent period.
6. The raise period.
7. The average yield of phages by the infected bacteria (average burst size).

Note the marked differences in the eclipse, the latent, and the raise periods between the phage T_4 and the mycobacteriophage D_{29}. For each system, the parameters of phage growth depend, in part, on the adsorption rate, growth rate of the host, the temperature of incubation, and the multiplicity of infection. It is important to note, however, that the differences between T_4 and D_{29} are characteristic of the phages themselves and independent of the mean division time of the hosts (about 20 minutes for *E. coli;* 2½ hours for *M. smegmatis*). The approximate latent period, eclipse period, and average burst size for some mycobacteriophages are given in Table 3.

TABLE 3. Approximate Latent Eclipse and Raise Periods, and Average Burst Size of Some Mycobacteriophages [data from literature]

Host-Mycobacteriophage	Latent Period (min.)	Eclipse Period (min.)	Rise Period (min.)	Average Burst
M. smegmatis 607 - D_{29}	65	55	30	70 - 125
M. smegmatis 607 - HP	180		60	
M. smegmatis 602 - B_1	65	52	20	100
M. smegmatis 9626 - D_{28}	70		30	65 - 116

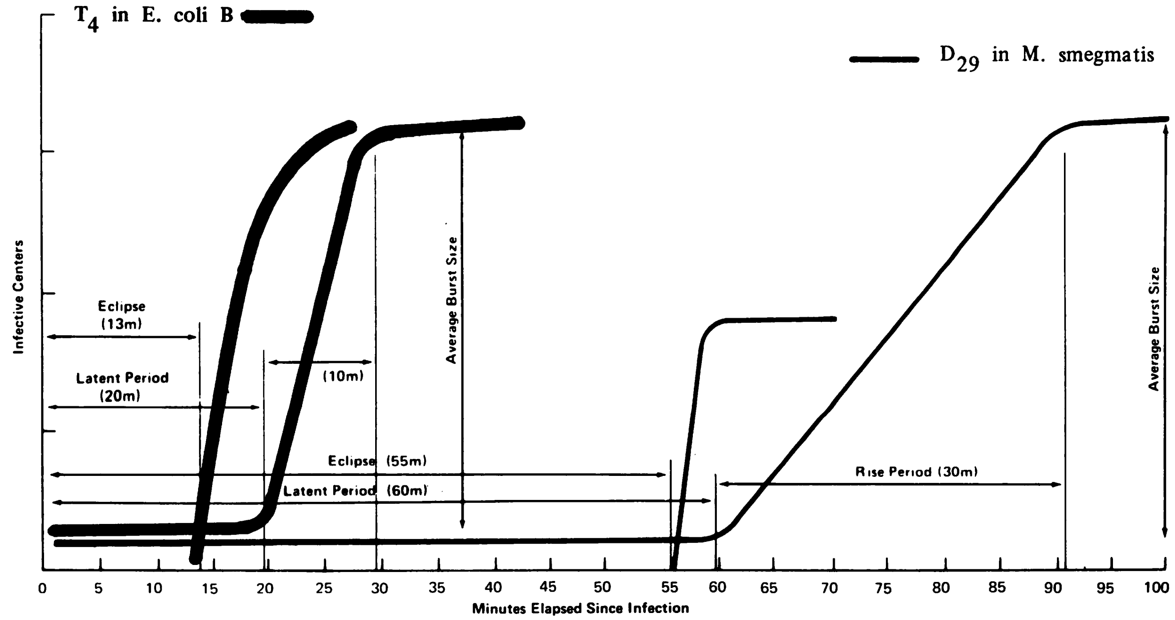

FIGURE 11. One-step growth of bacteriophage T_4 in *E. coli* B and of mycobacteriophage D_{29} in *M. smegmatis* ATCC 607 (original).

LYSIS

From the foregoing, it is clear that single phages behave as single particles with respect to plaque formation, and that the bacteria that adsorb several virus particles behave as if only one of the particles is effective. Clearly, for lysis to be judged by a loss in the culture's turbidity (clearing), it is necessary for almost all of the bacteria to be infected from the onset. Under the extreme dilutions the one-step growth must be measured, the fraction of the cells that lyse is a small fraction of the total bacterial population, and, therefore, clearing is apparent only after a few cycles of viral growth (*27*). For the kinetics of bacteriophage growth to be studied by observation of cell lysis (on the basis of clearing of a bacterial suspension), the phage input must be increased. Experiments with T_4 and *E. coli* showed that the culture lyses precisely at the end of the latent period; in contrast, experiments with D_{29} and *M. smegmatis* did not demonstrate appreciable loss in turbidity, well beyond the first cycle of viral replication. The behavior exhibited by mycobacteriophage D_{29} has been observed with other mycobacteriophages and lead us to hypothesize that these phages could be liberated without cell death (*51*). However, in our own laboratory, lysis of mycobacterial cells was seen by microscopic observation of individual bacteria, but characteristically, only a small fraction of the cells lyse within the time span of the first cycle of replication, even at high phage inputs, which may explain why clearing of the mycobacterial cultures is not usually observed. The factors underlining this phenomenon had not yet been elucidated. However, the following features of the mycobacteriophage growth may explain, in part, some of the present ambiguities:

1. The rate of adsorption of unadsorbed mycobacteriophage decreases with continued incubation, and is essentially independent of the phage input (*11*).
2. The fraction of bacteria producing phage seems to be a small fraction of the total bacteria, even at high multiplicities of infection (*57*).

SINGLE BURST

The burst size measured in the one-step growth is an average of the number of phages produced by all infected cells. Ellis and Delbrück (*33*) designed an experiment which makes it possible to determine the burst size in individual infected bacteria. The principle of the experiment is to dilute the bacteria in a one-step growth well before the end of the latent period, so that aliquots of the dilution distributed in many vials contain on the average less than one infected cell. (There is no such thing as a fraction of a cell; what is really meant is that some of the vials received no infected cells, while others received one, two, or more infected cells). The data shown in Table 4 is from a report concerning single bursts in the mycobacteriophage-mycobacteria system. In the experiment, the infected population was diluted and aliquots were dispensed in 72 vials. The vials were incubated long enough for the replication cycle to be completed, and then the contents of each vial were mixed with the indicator strain in overlay to determine the presence of infective phages and to estimate the number of infective centers in the vials that received at least one infected bacterium. The results showed that 15 of the plates had plaques, and 57 had no plaques. The presumed number of infected bacteria which each vial received can be inferred from these samples that show no plaques. When a random distribution of infected bacteria is assumed, the probability of a vial receiving *r* infected cells is given by the Poisson law,

$P_{(r)} = \frac{n^r}{r!} \cdot e^{-n}$, in which *n* is the average number of infected bacteria per vial. The value of *n* can be estimated from the number of plates with no plaques, which was 15 in the experiment.

Thus, $P_{(o)} = \frac{15}{72} = 0.79$. From the above equation:

$P_{(o)} = \frac{m^o}{o!} - e^{-n}$ $(n^o = 1; o\,! = 1)$, thence: $P_{(o)} = e^{-n}$ and $n = -\ln P_{(o)}$

for $P_{(o)} = 0.79$; $n = -\ln 0.79 = 0.24$

The average number of infected bacteria was, thus, 0.24. The probability of a vial receiving 1, 2, 3 or more infected cells is then:

$P_{(1)} = \frac{0.24^1}{1!} \cdot e^{-0.24} \times 0.79 = 0.1896$ (10% or 13.68 plates)

$P_{(2)} = \frac{0.24^2}{2!} \cdot e^{-0.24} = 0.0288 \times 0.79 = 0.022752$ (2% or 1.44 plates)

$P_{(3)} = \frac{0.24^3}{3!} \cdot e^{-0.24} = 0.002304 \times 0.79 = 0.0018$ (0.2% or 0.144 plates)

Thus, the calculated number of vials with one infected bacterium was 13, and the presumed number of vials with two infected bacteria was 2. Therefore, the total number of observed plaques (1121) was produced by 17 bacteria, or an average of about 1121 / 17 = 65 bacteriophages per bacterium. The wide fluctuation in the individual bursts reflects differences in the extent of phage multiplication inside individual bacteria.

PLATING EFFICIENCY

The efficiency of plating (E.O.P.) is defined as "the ratio of the plaque count under a given set of conditions to the plaque count under a standard set of conditions" (*90*). The conditions under which the one-step growth is measured are such that the probability of a bacterium adsorbing more than one phage particle is very small. (At time zero, the phages are added to an excess of bacteria, and then further adsorption is stopped by addition of antiserum and extreme dilution.) In the single-burst experiment, the fraction of samples without infected bacteria can be measured because only those samples that do not contain particles will not yield plaques.

From this fraction we have calculated the number of particles originally present in the suspension. The efficiency of plating was then estimated as the ratio of the above estimated value to the number of expected particles calculated from the phage input (ratio of item (e) to item (a), Table 4). The plating efficiency of the coli-phage T_2 and of the mycobacteriophage D_{28} were, respectively, 1.2 and 0.72. (The reader must have noticed that the high value of E.O.P. is in apparent contradiction with the previous discussion on the growth cycle of the mycobacteriophages and lysis of the host mycobacteria.)

In the above analysis, the E.O.P. of the same bacteriophage on the same host under two different sets of testing conditions was calculated. The E.O.P. of a bacteriophage on two or more hosts under the same testing conditions is a useful parameter in many experiments. For example, the titer of a suspension of mycobacteriophage D_{29} on *M. smegmatis* is much higher than on *M. phlei.* If the titer of *M. smegmatis* is equal to 1.0 (100%), the E.O.P. of *M. phlei* is determined by the ratio "titer on *M. phlei* / titer on *M. smegmatis.*"

TABLE 4. Single Burst Distribution
[data from references *63* and *86*]

	Bacteriophage T_2 - *E. coli*	**Mycobacteriophage D_{28} - *M. smegmatis***
a) Average number of infected bacteria per plate, calculated from phage input.	0.73	0.33
b) Total number of plates.	96	72
c) Expected number of plates without bursts.	96 x $e^{-0.73}$ = 46	72 x $e^{-0.33}$ = 57
d) Number of plates found without bursts.	39	57
e) Average number of infected bacteria per plate, calculated from (d).	ln(39/96) = 0.9	ln(57/72) = 0.24
f) Calculated number of plates with one burst.	96 x 0.9 x $e^{-0.9}$ = 35	72 x 0.24 x $e^{-0.24}$ = 13
g) Calculated number of plates with two bursts.	96 ($0.9^{2/2}$) x $e^{-0.9}$ = 16	72 ($0.24^{2/2}$) x $e^{-0.24}$ = 2
h) Calculated number of plates with three or more bursts.	96[1-(1+0.9+$0.9^{2/2}$)] x $e^{-0.9}$ = 6	72[1-(1+0.24+$0.24^{2/2}$)] x $e^{-0.24}$= (less than 1)
	Plaque count distribution 9 67 92 114 161 210 31 67 99 117 163 278 41 70 99 118 165 291 45 75 101 124 192 298 48 76 102 130 201 407 49 81 103 130 206 413 55 86 105 132 209 477 56 89 106 135 215 58 90 110 136 216 65 91 110 151 220	*Plaque count distribution* 20 115 25 126 27 133 36 141 37 51 73 76 98 101
Total count	7938	1121
Average yeld calculated from (e)	7938 / 96 x 0.9 = 92	1121 / 72 x 0.24 = 64
Efficiency of plating [(e)/(a)]	1.2	0.72

LYSOGENY

The literature on lysogenic (lysis-producing) mycobacteria is filled with ambiguities. In the opinion of this writer, most of the confusion that now exists can be traced to the improper description of virus multiplication. Most prominent is the almost total lack of quantitative data of the sort described earlier in this chapter. Thus, before attempting to review lysogeny in the mycobacteria, it seems pertinent to recall the experimental evidence upon which the following definition of a *lysogenic bacterium* is based: *"A lysogenic bacterium is a bacterium which perpetuates the capacity to form bacteriophages without intervention of exogenous bacteriophages"* (*65*). The definition indicates that the lysogenic bacterium has the hereditary capacity of producing bacteriophages; furthermore, it refers to a bacterium rather than to a bacterial population. The distinction is quite important, because a bacteriophage may become persistantly established in a population in certain circumstances ("Pseudolysogeny" or "Carrier state"). To Lwoff and Gutman (*65*), whose classic report is summarized below, "it was evident *a priori* that the study of 'mass cultures,' as it is usually practiced, can only furnish partial solutions to the problems of lysogenic bacteria. Only observations carried out on individual bacteria, or on microcultures containing a small number of individuals, are capable of leading to definite conclusions." The technical approach these investigators adopted was to transfer a single bacterial cell with the help of a micromanipulator to a liquid or a solid medium. In this manner, cultures derived from single cells can be obtained. The study was conducted with strain 899 of *Bacillus megaterium,* a strain whose cultures contained bacteriophages persistently. Observations on the cultures from single cells showed that the bacteria can multiply without the production of bacteriophages. However, the capacity to produce bacteriophages was maintained, and demonstrated either by mixing the bacteria with a sensitive indicator (in which case a plaque was formed) or by finding bacteriophages in microcultures where bacterial lysis was directly observed under the microscope. In certain experiments all the bacteria put in a little drop grew; in others, all the descendants lysed, and in some, only a fraction of the descendants were lysed. In many experiments in which a bacterium is transferred to a drop, then a dependent of this transferred to a second drop, and so on, an occasional cell may lyse and liberate bacteriophages. If the cells were artifically lysed with lysozyme, no bacteriophages were found. Finally, the bacteriophages liberated by lysis of one cell did not infect the remaining cells in the microculture.

In the report we summarized above, Lwoff enunciated the following three questions about lysogeny:

1. Can the faculty of producing bacteriophages really be perpetuated without intervention of exogenous bacteriophages?
2. How do lysogenic bacteria liberate the bacteriophages they produce?
3. If the production of bacteriophages pertains to only a certain proportion of the bacteria, then what factors induce the production of bacteriophages in a population of potentially lysogenic bacteria?

We have already seen how the first two questions were answered. In a second paper, Lwoff and his associates (*66*) showed that when a culture of *B. megaterium* in the exponential growth phase is irradiated with ultraviolet light, bacterial lysis begins within about 80 minutes after irradiation, the culture clears in 40 to 80 minutes, and bacteriophages are liberated. Lysis occurred only in cells that have grown in complex media. Thus, under certain conditions, all the bacteria of a lysogenic strain could be induced to lyse with production of bacteriophages.

These two reports of Lwoff and associates initiated considerable research on lysogeny, the discussion of which is beyond the scope of this book. However, those discoveries (reviewed in references *63* and *90*) that are pertinent to our discussion of lysogeny in the mycobacteria are listed here.

1. The lysogenic character is a hereditary property of a bacterium, that is, it is transmitted to its daughter cells.

2. In the lysogenic bacterium, the bacteriophage is in a state of prophage, that is, the bacteriophage DNA is intercalated in the bacterial cell's DNA.

3. At the time of induction, the prophage is released from the host's chromosome and initiates its vegetative replication which follows a course identical to the vegetative multiplication of an exogenous bacteriophage in a sensitive bacterium, that is, there is an eclipse period, a latent period, a period of maturation, the rise period, and finally the virion is liberated by lysis of the host. Lysis always precedes the liberation from the host bacterium.

4. The lysogenic bacterium is immune to exogenous infection by the homologous bacteriophage.

    a. Lysogenic bacteria adsorb the homologous phage, in contrast to resistant mutants.

    b. The superinfecting homologous phage infects its DNA, but the newly introduced genes neither function nor replicate.

    c. Immunity is specific and is determined by the phages.

5. Some bacteriophages always give a lytic response (virulent phages); others can give either a lytic (productive) or lysogenic (reductive) response (temperate phages).

6. Only the temperate phages can give a lysogenic response.

7. The plaques of temperate phages are turbid because some bacteria become lysogenic and immune. Virulent phages produce clear plaques.

8. Titration of temperate phages gives lower counts than the actual number of virions, because some of the bacteria become lysogenic.

9. The temperate phage genome contains genes that control the synthesis of a cytoplasmic immunity repressor.

10. The bacterial chromosome contains one or more prophage attachment sites.

11. The repressor prevents the autonomous replication of the phage, but not the replication of the integrated prophage which is under the control of the host-cell replication mechanism.

12. Lysogeny is under the genetic control of the phage genes. Mutation in these genes result in the loss of the ability to lysogenize. These mutants form clear plaques (*c* mutants); the region in the chromosome concerned with the maintenance of lysogeny is thence called the *c* region. Three genes have been recognized in this segment of the phage chromosome: c_I, c_{II}, and c_{III} genes.

Now the information available on lysogeny in the mycobacteria must be analyzed. Although there is no reason to doubt the existence of lysogenic mycobacteria, the question that must be asked beforehand is whether lysogeny was ever experimentally demonstrated.

The occurrence of lysogeny in the mycobacteria cannot be doubted. However, reports on lysogeny may instead prove to be reports of a persistent viral infection of the bacterial population. Indeed, some of the literature indicates a high frequency of "cured" cells either spontaneous or after treatment of the bacterial population with bacteriophage antiserum and Tween 80 (*4, 49, 108*), and the presence of high bacteriophage titers (*108*). These observations lead to the supposition that the relationship between the bacteriophage and the bacterial population is one of a "carrier state" rather than of lysogeny (*6*). Other investigators have reported that treatment of the bacterial population with antiserum or Tween 80 failed to eliminate the bacteriophages (*10, 81*). The titer of virus produced spontaneously in the cultures was low. Furthermore, the infected bacteria were immune to the homologous bacteriophage.

The majority of papers on the lysogeny in the mycobacteria are difficult to interpret, mainly because the authors assume rather than demonstrate lysogeny. Instead of a list of all reports so far published on the subject, a summary is presented of perhaps the first report in which the lysogenic state was satisfactorily demonstrated — that of Bowman and Redmond (*10*).

Bowman and Redmond showed that a strain of *M. butyricum* propagated in the laboratory for many years, produced spontaneously free bacteriophages that replicated lytically on the indicator strain *M. smegmatis* 607. The bacteriophage was named R_1 and the host strain *M. butyricum* (R_1) according to the common practice of labeling lysogenic bacteria. All isolated colonies of *M. butyricum* (R_1) were capable of producing bacteriophage and in a bacterial population; the frequency of bacteria producing virus spontaneously was about 1×10^{-6}. In other words, although all bacteria were capable of producing phage as evidenced by cloning, only in a few bacteria in the population did induction occur spontaneously. However, the fraction of bacteria yielding bacteriophage increased several times after UV-irradiation.

When bacteriophage R_1 was recovered from the indicator strain and was then spotted onto *M. butyricum* (R_1), no plaques were produced, indicating that the host organism was immune to infection by the R_1 phage. The following were then the criteria presented to support the lysogenic state: the bacteriophage was part of the genetic structure of the bacteria existing in the prophage state; the bacteria were immune to superinfection with the same bacteriophage; occasionally, the bacteriophage was spontaneously induced to replicate vegetatively in a bacterium; and vegetative replication was induced by ultraviolet light.

BACTERIOPHAGE TYPING

Although typing of the mycobacteria by bacteriophages has been reported by several investigators (for a review, see Redmond, *75*), this did not become a laboratory tool for two main reasons. One is that the bacteriophage-bacterium relationships are not well understood and are therefore difficult to control, and the other is that procedures have not been standardized so that proposed typing systems can be tested for their diagnostic or medical relevance.

Recently, Takeya and Tokiwa (*96*) reported that some mycobacterial strains were bacteriocinogenic and proposed a typing system of rapidly growing mycobacteria. Like bacteriophage typing, the use of mycobacteriocins for typing purposes needs to be fully investigated.

Drug Resistance

INTRODUCTION

Drug-resistance is the ability of a bacterium to survive the exposure to a drug at a concentration that inhibits or kills the parental cells, and to transfer the character to its progeny. In *M. tuberculosis* the only known mechanism for a bacterium to acquire resistance is spontaneous mutations, which, as we have discussed before, happen at extremely low frequencies. In this instance, the shift of a predominantly sensitive population into a population predominantly composed of resistant bacteria requires the introduction in the system of the selective force of the drug. There are other mechanisms, however, whereby bacteria may become resistant. Thus, a bacterium may receive a resistance gene from a donor resistant bacterium by essentially three mechanisms: conjugation, transduction, and transformation. To date there is no evidence indicating that the transfer of genetic material plays a role in drug-resistance in tuberculosis.

The case in other pathogenic mycobacteria is altogether distinct. These organisms exhibit multiple drug-resistance, which have the following characteristics. The bacterial populations are heterogeneous in respect to their susceptibility to any given drug, and the proportion of drug-resistant bacteria shows wide variations not only when strains are compared but also in single strains tested at intervals. The nature of these variations in the genetic properties of the bacterial populations has not been clarified so far, but could be caused by:

1. Mutator effects.
2. Resistance transfer factors.
3. Inducible drug-resistance.
4. Selection.

MUTATOR EFFECTS

Some bacterial strains contain mutator genes that increase the mutation rates of other genes (*8, 41, 44, 55, 56, 61, 69, 89, 98*). The mutator genes appear to affect the DNA repair mechanisms in the cell. The deficiency in the repair mechanisms lead to high mutability, to increased sensitivity to agents that damage DNA, such as UV radiation, and to defective recombination. On the other hand, radiation sensitive mutants (*UVr*⁻ mutants are deficient in excising pyrimidine dimers; *rec*⁻ mutants are deficient in recombination; *lex*⁻ mutants have normal recombination efficiency; and the *ras*⁻ mutants are sensitive to UV light but only slightly to x-irradiation, have normal recombination ability and show an abnormally high frequency of UV-induced mutation) may also exhibit high mutation rates.

The frequency of colony type variation in *M. avium* (transparent → opaque variation) was reported to be 10^{-4} - 10^{-5} (*67*). This rate of variation is unusually high, and if caused by mutations, then the strain studied could carry a mutator gene. The finding of mutator genes in *M. avium* could then explain the extreme variation that these bacteria exhibit in regard to drug-resistance. Studies on the UV-sensitivity of *M. avium* and other mycobacteria (*25, 67a*), and the finding that these bacteria are capable of repairing the UV-induced damage of DNA do not appear to substantiate the possibility that the high variability in drug-resistance in these bacteria might be caused by mutator effects.

RESISTANCE TRANSFER FACTORS

Since mutations occur spontaneously and independently of each other, a bacterium becomes resistant to one drug at a time. In 1950, during an epidemic of bacillary dysentery in Japan (*105*), strains exhibiting multiple drug-resistance were isolated. Further studies on multiple drug resistance in the Enterobacteriaceae and other bacteria showed that in most clinical instances multiple drug-resistance in these bacteria is specified by extrachromosomal or satellite DNA's referred to as drug resistance factors or R-factors. The R-factors are capable of autonomous replication, and can be transferred into a recipient bacterium either by conjugation or transduction. In the strains that transfer the R-factor by conjugation, it appears that the R-factor contains two determinants: the transfer factor (RTF) and the genes that are specific for drug resistance (*105*). When a bacterium carrying a RTF is introduced in a population of sensitive bacteria, these become rapidly resistant as a consequence of the transfer of the R-factors.

Evidence for the occurrence of a R-factor in *M. smegmatis* was recently published (*52*), and the marker Str^r was successfully transduced from *M. smegmatis* into *M. tuberculosis* (*53*). The significance of these observations insofar as the problem of multiple resistance in the mycobacteria is concerned, is difficult to assess at the present time.

INDUCIBLE DRUG-RESISTANCE

Resistance that is caused by extrachromosomal genes is, in most cases, mediated by enzymes that inactivate the drug. The synthesis of the enzymes may be constitutive or inducible. In the case of the penicillinase in the staphylococci, the penicillinase region may be carried in a plasmid or in the chromosome, and the synthesis of the enzyme is inducible (*78*). Inducible resistance seemingly unrelated to a plasmid and to enzymatic inactivation was reported in *S. aureus* (*106*). Cells grown in the absence of erythromycin are sensitive, but when exposed to low concentrations of the antibiotic virtually every cell in the population becomes resistant within about 1 hour. Strains induced by low concentrations of erythromycin may become resistant to other macrolides (*107*).

Resistance to streptomycin in a R$^+$ strain of *M. smegmatis* was found to be inducible and caused by the inactivation of the antibiotic (*52*). Except in this case, attempts in this laboratory to demonstrate inducible drug-resistance in other mycobacteria have been unsuccessful. (See Chapter 3 for a discussion of induction.)

SELECTION

When *M. avium* cultures in a liquid medium are plated on solid medium at regular intervals the frequency of transparent, opaque and rough colonies varies widely. These variants differ not only in their susceptibility to a variety of drugs but also in their pathogenecity for chickens and mice (*32, 71, 85*). The high frequency of variation (10^{-4} - 10^{-5}) (*67a*) does not appear to be caused by mutator effects (see above) and could perhaps be due to a selection mechanism of the kind described by Braun and his associates (*15*), as described earlier in this chapter.

In spite of its considerable interest and medical importance, the nature of multiple drug-resistance in the mycobacteria has not been satisfactorily investigated.

REFERENCES

1. Atwood, K. C., Schneider, L. K., and Ryan, F. J. (1951) Cold Spring Harb. Symp. Quant. Biol. 16:345.
2. Armstrong, A. R. (1960) Amer. Rev. Resp. Dis. 81:498.
3. Armstrong, A. R. (1965) Amer. Rev. Resp. Dis. 91:440.
4. Baess, I. (1969) Acta Pathol. Microbiol. Scand. 76:464.
5. Baess, I., and Jentzen, M. W. (1969) Acta Pathol. Microbiol. Scand. 75:331.
6. Baess, I. (1971) Acta Pathol. Microbiol. Scand., Section B, 79:428.
7. Beggs, W., and Jenne, J. W. (1970) Infect. Imm. 2:479.
8. Böhme, H. (1967) Biochem. Biophys. Res. Comm. 28:191.
9. Bourgeois, P., Dubois-Verliere, M., and Macl, M. (1958) Rev. Tuberc. (Paris) 22:108.
10. Bowman, B. W. and Redmond, W. B. (1958) Amer. Rev. Resp. Dis. 80:232.
11. Bowman, Jr., B. U. (1958) J. Bacteriol. 76:52.
12. Bowman, Jr., B. U. and Redmond, W. B. (1959) Amer. Rev. Resp. Dis. 80:232.
13. Bowman, B. U. (1969) Microbios. 4:347.
14. Bowman, B. U., Newman, H. A. I., Moritz, J. M. and Kochler, R. M. (1973) Amer. Rev. Resp. Dis. 407:42.
15. Braun, W. (1965) "Bacterial Genetics," W. B. Sanders Co., Philadelphia, 1965.
16. Braunstein, S. N., and Franklin, R. M. (1971) Virology 43:685.
17. Castelnuovo, G., Belezza, G. and Yamanaka, S. (1970) Ann. Inst. Pasteur 119:302.
18. Castelnuovo, G. (1970) in "Host-virus relationships in *Mycobacteria, Nocardia* and *Actinomyces,*" edited by Juhasz, S. E. and Plummer, G., Charles C. Thomas, Publisher, Springfield, Ill., 1970.
19. Cater, J. C. and Redmond, W. B. (1961) Canad. J. Microbiol. 7:697.
20. Cavalli-Sforza, L. L. and Lederberg, J. (1955) Genetics 41:367.
21. d'Herelle, F. (1921) "Le Bacteriophage: son role dans 'immunite' ", Masson et C^{ie}, ed., Paris, 1921.
22. David, H. L. (1970) Appl. Microbiol. 20:810.
23. David, H. L. and Newman, C. M. (1971) Amer. Rev. Resp. Dis. 104:508.

23a. David, H. L., Newman, C. and Jones, W. D., Jr. (1971) Appl. Microbiol. 4:318.

24. David, H. L. (1973) Appl. Microbiol. 21:888.
25. David, H. L. (1973) Amer. Rev. Resp. Dis. 108:1175.
26. Davis, B. D., Dulbecco, R., Eison, H. N., Ginsberg, H. S. and Wood, Jr., W. B. (1968) "Microbiology" Harper & Row, Publishers, New York, Evanston, London, 1968.
27. Delbrück, M. (1940) J. Gen. Physiol. 23:643.
28. Demerec, M. (1948) J. Bacteriol. 56:63.
29. Doermann, A. H. (1948) J. Bacteriol. 55:257.
30. Doermann, A. H. (1952) J. Gen. Physiol. 35:645.
31. Dubos, R. J. (1945) Proc. Soc. Exptl. Biol. Med. 58:361.
32. Dunbar, F. P., Pejovic, J., Cacciatore, R., Peric-Golia, L. and Runyon, E. H. (1968) Scand. J. Resp. Dis. 49:153.
33. Ellis, E. L. and Delbrück, M. (1939) J. Gen. Physiol. 22:365.
34. Fay, D. D. and Bowman, B. U. (1971) Bacteriol. Proc., p. 174.
35. Fenner, F., Martin, S. P. and Pierce, C. H. (1949) Ann. N. Y. Acad. Sc. 52:751.
36. Fenner, F. (1951) Amer. Rev. Tub. 64:353.
37. Fregnan, G. B., Randall, H. M. and Smith, D. W. (1962) J. Bacteriol. 83:828.
38. Froman, S. et al. (1954) Amer. J. Pub. Health 44:1326.
39. Gardner, G. M. and Weiser, R. S. (1947) Proc. Soc. Exptl. Biol. Med. 66:205.
40. Gelbart, S. M. and Juhasz, S. E. (1970) J. Gen. Microb. 64:253.
41. Goldstein, A. and Smoot, J. S. (1955) J. Bacteriol. 70:588.
42. Murohasi, T., Tokunaga, T. and Seki, M. (1961) Acta Tub. Penumol. Scand. 41:33.
43. Hayes, W. (1964) "The Genetics of Bacteria and Their Viruses." John Wiley & Sons, Inc., New York, 1964.
44. Helling, R. B. (1968) J. Bacteriol. 96:975.
45. Hnatko, S. I. (1953) Canad. J. Med. Sc. 31:462.
46. Hoffman-Berling, H. (1964) Virology 22:305.
47. Hsie, J. Y. and Bryson, V. (1950) Amer. Rev. Tub. 62:286.

48. Imaeda, T. and San Blas, F. (1969) J. Gen. Virol. 5:493.
49. Jones, W. D., Jr. and White, A. (1968) Canad. J. Microbiol. 14:551.
50. Jones, W. D., Jr. and David, H. L. (1970) Amer. Rev. Resp. Dis. 102:814.
51. Jones, W. D., Jr. and David, H. L. (1971) Amer. Rev. Resp. Dis. 103:618.
52. Jones, W. D., Jr. and David, H. L. (1970) Tubercle 53:35.
53. Jones, W. D., Jr., Beam, R. E. and David, H. L. (1974) Tubercle 55:73.
54. Juhasz, S. E. and Bonicke, R. (1965) Canad. J. Microbiol. 11:235.
55. Jyssum, K. (1960) Acta Pathol. Microbiol. Scand. 48:113.
56. Jyssum, K. (1968) J. Bacteriol. 95:165.
56a. Kelner, A. (1949) J. Bacteriol. 58:511.
57. Kölbel, H. (1970) in "Host-virus relationships in *Mycobacteria, Nocardia* and *Actinomyces,*" edited by Juhasz, S. E. and Plummer, G., Charles C. Thomas Publisher, Springfield, Ill., 1970.
57a. Kölbel, H. K. and Mohelska, H. (1971) Zbl. Bakt. Hyg. I. Abt. Orig., 217:507.
58. Konickova-Radochova, M. and Malek, J. (1969) Folia Microbiol. 14:201.
59. Konickova-Radochova, M. and Malek, J. (1969) Folia Microbiol. 14:251.
60. Lederberg, J. and Lederberg, E. (1952) J. Bacteriol. 63:399.
61. Liberfarb, R. M. and Bryson, V. (1970) J. Bacteriol. 104:363.
62. Lincoln, R. E. (1947) J. Bacteriol. 54:745.
63. Luria, S. E. and Darnell, Jr., J. E. (1967) "General Virology," John Wiley & Sons, Inc., New York, London, Sydney, 2nd Ed., 1967.
64. Luria, S. E. and Delbrück, M. (1943) Genetics 28:491.
65. Lwoff, A. and Gutman, A. (1950) Ann. Inst. Past. 78:711.
66. Lwoff, A., Siminovitch, L. and Kjeldgaard, N. (1950) Compt. Rend. Sc. Acad. Sciences (France) 231:190.
67. McCarthy, C. (1970) Infect. Imm. 2:223.
67a. McCarthy, C. M. and Schaefer, J. O. (1974) Appl. Microbiol. 28:151.
68. Mitchison, D. A. (1954) Brit. Med. J. 10:115.
69. Miyake, T. (1960) Genetics 45:11.
70. Mizuguchi, Y. and Tokunaga, T. (1971) Jap. J. Microb. 15:359.
71. Moehring, J. M. and Solotorowsky, M. R. (1965) Amer. Rev. Resp. Dis. 92:704.
72. Nakamura, R. M., Tokunaga, T. and Murohashi, T. (1967) Amer. Rev. Resp. Dis. 96:542.
73. Pettitjohn, D. and Hanawalt, P. (1964) J. Mol. Biol. 9:395.
74. Piquet, J. D. (1960) Thesis, Univ. of Lausanne, Faculte de Sciences, in Redmond, W. B. (1963).
75. Redmond, W. B. (1963) Adv. Tuberc. Res. 12:191.
76. Redmond, W. B. and Cater, J. C. (1960) Amer. Rev. Resp. Dis. 82:781.
77. Redmond, W. B. and Ward, D. M. (1966) Bull. W.H.O. 35:563.
78. Richmond, M. H. and Johnston, J. (1969) in "Bacterial episomes and plasmids," A Ciba Foundation Symposium, ed. by G. E. W. Wolstenholme and M. O'Connor, Little, Brown and Co., Boston, p. 179.
79. Roberts, R. B. and Aldous, E. (1949) J. Bacteriol. 57:363.
80. Robins, M. L., Amako, K., Muraoka, S. and Takeya, K. (1970) in "Host-virus relationships in *Mycobacteria, Nocardia* and *Actinomyces,*" edited by Juhasz, S. E. and Plummer, G., Charles C. Thomas, Publisher, Springfield, Ill.
81. Russell, R. L., Jann, F. J. and Froman, S. (1960) Amer. Rev. Resp. Dis. 82:384.
82. Sager, R. and Ryan, F. J. (1962) "Cell Heredity" John Wiley and Sons, Inc., New York.
83. Salivar, W. O., Tzagaloff, H. and Pratt, D. (1964) Virology 24:359.
84. Saroja, D. and Gopinathan, K. P. (1973) Ant. Ag. Chemoth. 4:643.
85. Schaefer, W. B., Davis, C. L. and Cohn, M. L. (1970) Amer. Rev. Resp. Dis. 102:1970.
86. Sellers, M. I., Baxter, W. L. and Runnals, H. R. (1962) Canadian J. Microbiol. 8:389.
87. Sellers, M. I. and Tokunaga, T. (1966) J. Exp. Med. 123:327.
88. Sellers, M. I. and Tokunaga, T. (1970) in "Host-virus relationships in *Mycobacteria, Nocardia* and *Actinomyces,*" edited by Juhasz, S. E. and Plummer, G., Charles C. Thomas, Publisher, Springfield, Ill.
88a. Sellers, M. I., Nakamura, R. and Tokunaga, T. (1970) J. Gen. Virol. 7:233.
89. Siegel, E. and Bryson, V. (1967) J. Bacteriol. 94:38.

90. Stent, G. S. (1963) "Molecular Biology of Bacterial Viruses," W. H. Freeman and Co., S. Francisco, London.
91. Stocker, B. A. D. (1949) J. Hyg. 47:398.
92. Sundar Raj, C. V. and Ramakrishnan, T. (1970) Nature 228:280.
93. Sundar Raj, C. V. and Ramakrishnan, T. (1971) J. Indian Inst. Sc. 53:126.
94. Szybalsky, W. and Bryson, V. (1952) Amer. Rev. Tub. 65:768.
95. Tacquet, A., Tison, F. and Devulder, B. (1965) Ann. Inst. Past. 108:514.
96. Takeya, K. and Tokiwa, H. (1972) Int. J. Syst. Bacteriol. 22:178.
97. Tokunaga, T. and Sellers, M. I. (1964) J. Exptl. Med. 119:139.
98. Treffers, H. P., Spinelli, V. and Belser, N. O. (1954) Proc. Nat. Acad. Sci. 40:1064.
99. Tokunaga, T. and Sellers, M. I. (1965) J. Bacteriol. 89:1965.
100. Tokunaga, T. and Nakamura, R. M. (1968) J. Virol. 2:110.
101. Tokunaga, T., Sellers, M. I. and Furuchi, I. (1970) in "Host-virus relationships in *Mycobacteria, Nocardia* and *Actinomyces,*" edited by Juhasz, S. E. and Plummer, G., Charles C. Thomas Publisher, Springfield, Ill.
102. Tokunaga, T., Mizuguchi, Y. and Suga, K. (1973) J. Bacteriol. 113:1104.
103. Tsukamura, M., Noda, Y., Hayashi, M. and Yamamoto, M. (1954) Jap. J. Microbiol. 3:1.
104. Tsukamura, M. (1961) J. J. Tub. 9:43.
105. Watanabe, T. (1963) Bact. Rev. 27:87.
106. Weaver, J. R. and Pattee, P. A. (1964) J. Bacteriol. 88:574.
107. Weisblum, B. and Demohn, V. (1969) J. Bacteriol. 98:447.
108. White, A., Foster, F. and Lyon, L. (1962) J. Bacteriol. 84:815.
109. Woodley, C. L., Kilburn, J. O., David, H. L. and Silcox, V. A. (1972) Antimicrob. Ag. Chemoth. 2:245.
110. Yegian, D. and Vanderlinde, P. J. (1948) J. Bacteriol. 56:177.
111. Youmans, G. P. and Youmans, A. S. (1949) J. Bacteriol. 58:247.

CHAPTER 5

Mechanisms of Action of Antituberculosis Drugs

Introduction

Investigations on the mechanisms of action of the antituberculosis drugs are intimately related to investigations on their pharmacologic properties and to investigations on the physiological bases of resistance and ways to circumvent it. An understanding of these actions at the biochemical level, particularly the isolation and characterization of the target enzyme(s), may eventually lead to the synthesis of derivatives that are less toxic to humans and it may lead to the synthesis of derivatives that inhibit the growth of resistant mutants. Unfortunately, there is very little information on the mechanisms of action of antituberculosis drugs. In this chapter, the mechanisms of action of isoniazid, streptomycin, D-cycloserine, rifampin and ethambutol are discussed.

Isoniazid

Early hypotheses to explain the mode of action of isoniazid were reviewed by Goldman (*9*). At present there are two major views. One is that isoniazid combines with an inhibitor of NADase which is present in cell free extracts of *M. tuberculosis.* Consequently, NADase degrades NAD and the metabolism of the cells is critically deranged (*22, 23*). The second is that isoniazid specifically inhibits the biosynthesis of mycolic acids which could cause a critical change in the cell's envelope (*19*) or some other still unclear lethal lesion.

The inhibition of mycolate synthesis is of great interest in this regard because even though isoniazid inhibits the synthesis of other cellular compounds and enzymatic reactions, it does so at concentrations higher than the minimal inhibitory concentrations and later than cell death begins to occur. In contrast, inhibition of mycolate synthesis is the earliest event in cells exposed to the drug and complete inhibition of mycolate synthesis precedes cell death (*15*). Furthermore, mycolate synthesis can be partially restored in cells exposed to the drug for less than 10 hours, and the restoration of mycolate synthesis is in close parallel to cell viability (*16*). Careful quantitative studies by Wang and Takayama (*17*) indicated that the rate of inhibition of mycolate synthesis is concentration dependent. The intracellular concentration of isoniazid which causes 50% inhibition of mycolate synthesis was calculated to be 9 μM.

Although the ultimate cause of death in *M. tuberculosis* treated with isoniazid remains obscure, the inhibition of mycolate synthesis is possibly the primary site of action of the drug. Indeed, 1) inhibition of mycolate synthesis is consistent with the specificity of the drug; 2) inhibition occurs very early; 3) 50% inhibition is apparent at a concentration of 9 μM, whereas, the inhibitiȯn of NADase inhibitor requires 0.5 mM (*12*); and 4) complete inhibition of mycolate synthesis precedes cell death, and 5) the restoration of mycolate synthesis parallels cell viability.

Streptomycin

Streptomycin is a known inhibitor of protein synthesis. Although streptomycin inhibits protein synthesis in *M. tuberculosis* and other mycobacteria, there are no detailed investigations of its mode of action in these organisms. Studies *in vitro* of synthesis of polyphenylalanine in reaction mixtures containing polyuracil, ribosomes and radioisotope labeled phenylalanine indicated that resistance to streptomycin in *M. smegmatis* was associated with the 30 S ribosomal subunit (*21*).

D - cycloserine

M. tuberculosis is sensitive to very low concentrations of D-cycloserine. The ultimate cause of death is cell lysis[1]. Both growth inhibition and cell lysis are antagonized by D-alanine (*10, 24*). The growth inhibitory effect D-cycloserine exerts upon other mycobacteria is also reverted by D-alanine (*10*).

D-cycloserine is a competitive inhibitor of alanine racemase and of D-alanyl-D-alanine synthetase, enzymes that catalyze respectively the conversion of L-alanine to D-alanine and of D-alanine to D-alanyl-D-alanine (*30*). The synthesis of the dipeptide is necessary in the biosynthesis of cell walls in the bacteria and it is transferred to the UDP-acetyl-muramyl-tripeptide precursors to form the pentapeptide precursor for the mucopeptide in the cell walls. Because D-cycloserine blocks the synthesis of the D-alanine dipeptide, cells exposed to the drug accumulate the incomplete precursor UDP-acetylmuramyl tripeptide.

Studies in *M. tuberculosis* demonstrated the occurrence in cell free extracts of D-alanyl racemase and D-alanine synthetase (*1*). The antibiotic competitively inhibited the synthetase enzyme, and a Km for D-alanine of 2 x 10^{-3} M and a Ki for D-cycloserine of 3 x 10^{-5} M were established. Because these parameters were similar to those obtained for the enzymes of bacteria less sensitive to the antibiotic, it was concluded that the unusually high susceptibility of the tubercle bacilli could not be explained solely on the basis of its action on the synthetase. Further analysis indicated that the high sensitivity of the tubercle bacilli to the antibiotic may be associated to a low cellular concentration of the enzyme (*4*). Competitive experiments showed that D-cycloserine is actively transported, and that the same system transports L-alanine, D-alanine, glycine, D-serine, and O-carbamyl serine. These investigations lead to the conclusion that the high susceptibility of the tubercle bacilli to D-cycloserine is the product of the sensitivity of the synthetase, a low synthetase enzyme concentration and an active transport system.

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[1] See Chapter 2 for a discussion of cell lysis.

D-cycloserine induces the accumulation of an incomplete precursor of the cell wall mucopeptide in *M. tuberculosis* and in *M. smegmatis* (*2, 11*). In both cases the intermediate precursor was identified as UDP-glycolylmuramyl-L-alanyl-γ-D-glutamyl-meso-α,α'-diaminopimelic acid (*11, 14*).

Inhibitors of D-alanine racemase, such as O-carbamyl-D-serine and D-serine, also cause the accumulation of acylamino sugars in *M. tuberculosis* and *M. smegmatis* (*3, 20*) and induce cell lysis. The nature of the acylamino sugars whose accumulation is induced by O-carbamyl-D-serine was not investigated; D-serine in *M. smegmatis* was shown to induce the accumulation of a UDP-glycolylmuramyl tripeptide.

In addition, to inhibiting the biosynthesis of the cell wall mucopeptide in the tubercle bacilli, D-cycloserine also causes an inhibition of the synthesis of the Wax D peptidoglycolipid (*2*). The inhibition of Wax D synthesis was apparent at concentrations of the drug lower than the concentration necessary to cause the inhibition of the wall mucopeptide. The significance of this observation as far as the lethal effect of the antibiotic is concerned is not clear.

Rifampin

The mechanism of action of rifampin was investigated in *M. smegmatis* (*18*). Like in other bacteria, rifampin was shown to inhibit the DNA-dependent RNA polymerase. Rifampin inhibits the incorporation of uracil into trichloroacetic acid insoluble material in *M. tuberculosis* (*6*) and in *M. kansasii* (*5*). Therefore, the drug inhibits protein synthesis at the transcription level.

Ethambutol

Ethambutol was shown to inhibit the synthesis of DNA, RNA and protein (*7, 8*). The primary site of action of the drug remains undecided.

REFERENCES

1. David, H. L., Takayama, K. and Goldman, D. S. (1969) Amer. Rev. Resp. Dis. 100:579.
2. David, H. L., Takayama, K. and Goldman, D. S. (1969) Infect. Imm. 1:74.
3. David, H. L. (1970) Amer. Rev. Resp. Dis. 102:68.
4. David, H. L. (1971) Appl. Microbiol. 21:888.
5. David, H. L. (1974) J. Bacteriol. 119:527.
6. Dickinson, J. M., Jackett, P. S. and Mitchison, D. A. (1972) Amer. Rev. Resp. Dis. 105:519.
7. Forbes, M., Kuck, N. A. and Peets, E. A. (1962) J. Bacteriol. 84:1099.
8. Forbes, M., Kuck, N. A., and Peets, E. A. (1965) J. Bacteriol. 89:1299.
9. Goldman, D. S. (1959) Adv. Tuberc. Res. 11:1.
10. Hawkins, J. and McClean, V. R. (1966) Amer. Rev. Resp. Dis. 93:594.
11. Petit, J. F., Adam, A. and Wietzenbin-Falszpan, J. (1970) FEBS Letters 6:55.
12. Srinakash, K. S. and Ramakrishnan, T. (1969) Indian J. Biochem. 6:49.
13. Strominger, J. L., Ito, E. and Threnn, R. H. (1960) J. Am. Chem. Soc. 82:998.
14. Takayama, K. David, H. L., Wang, L. and Goldman, D. S. (1970) Biochem. Biophys. Res. Comm. 39:7.
15. Takayama, K., Wang, L. and David, H. L. (1972) Antim. Ag. Chemoth. 2:29.
16. Takayama, K., Armstrong, E. L. and David, H. L. (1974) Amer. Rev. Resp. Dis. 110:43.
17. Wang, L. and Takayama, K. (1972) Antim. Ag. Chemoth. 2:438.
18. White, R. J., Lancini, G. C. and Silvestri, L. G. (1973) J. Bacteriol. 108:737.
19. Winder, F. G. and Collins, P. B. (1970) J. Gen. Microbiol. 63:41.
20. Yabu, K. and Huempfner, H. R. (1974) Antim. Ag. Chemoth. 6:1.
21. Yamada, T., Kunitsugu, M., Shoji, K. and Hori, M. (1974) Antim. Ag. Chemoth. 6:46.
22. Youatt, J. (1969) Amer. Rev. Resp. Dis. 99:729.
23. Youatt, J. and Tham, S. H. (1969) Amer. Rev. Resp. Dis. 100:31.
24. Zygmunt, W. A. (1963) J. Bacteriol. 85:1217.

part 2

Medical Mycobacteriology

Introduction

Nosology is the branch of medicine concerned with the classification of diseases. The diseases caused by bacteria of the genus *Mycobacterium* are designated *mycobacterioses*. We will be concerned only with the human mycobacterioses, two of which (tuberculosis and leprosy) have been known for centuries. During the last two decades, six other mycobacterioses were recognized. These lately described diseases have not been given universally accepted designations yet, and they are generally referred to as atypical tuberculosis or atypical mycobacterial infections. These designations are not satisfactory, since tuberculosis is very polymorphic in its pathology, clinical progression, and outcome. Actually, the term "atypical" has been borrowed from the bacteriologists, themselves struggling with it for some time with much discomfort.

The progress already made in the bacteriology of the mycobacteria makes it possible to classify these diseases in relation to their specific etiologic agents. For didactic purposes, this author has adopted the following nomenclature: the word mycobacteriosis followed by the specific epithet indicates the disease; when infection rather than disease is to be indicated, the species is followed by the word infection. For example, the disease caused by *M. scrofulaceum* is called mycobacteriosis *scrofulaceum*, whereas infection with the same organism as judged, for example, by a skin test reaction with PPD-G is called *M. scrofulaceum* infection. Tuberculosis and leprosy are named by these accepted designations. Table 1 is a list of the etiologic agents of the human mycobacterioses, arranged in relation to their clinical significance. The *strict pathogens* are those mycobacteria whose identification in clinical specimens means the presence of actual disease for all practical purposes. Potential pathogens that are isolated may or may not be responsible for the actual pathological process. The pathogens classified as rare are mycobacteria that are isolated from human secretions much more often than they are found to cause disease. These designations are used as a matter of convenience, and the reader must not assume that they are generally accepted.

TABLE 1. Mycobacteria Pathogenic for Man

Strictly Pathogenic	**Potential Pathogenic**	**Rarely Pathogenic**
M. leprae	*M. kansasii*	*M. scrofulaceum*
M. tuberculosis	*M. marinum*	*M. fortuitum*
M. bovis	*M. avium-intracellulare*	
M. ulcerans	*M. xenopi*	
M. africanum[1]	*M. simiae*[1]	
	M. szulgai[1]	

[1] Species of undefined taxonomic status.

CHAPTER 1

The Mycobacterioses

Mycobacterium tuberculosis

INTRODUCTION

Although pulmonary tuberculosis is the most frequent form of the disease in humans, tuberculosis lesions can be found in any organ or tissue. Thus, the tubercle bacilli caused such a wide variety of lesions and clinical symptoms that only in 1819 was it recognized by Laennec that various clinical conditions could pertain to a common pathological process, a notion that was confirmed by the discovery of the tubercle bacilli by R. Koch in 1882. Some twenty years earlier Villemin had demonstrated that the caseous material from the human tuberculous lung could produce tuberculosis in the rabbit, thus proving the infectious nature of the disease. Koch's work on the etiology of tuberculosis is a landmark in the history of medical bacteriology. He established the criteria for considering an organism to be the etiological agent of an infectious disease. These criteria, henceforth known as the *Koch's postulates*, are described by him as follows: "In order to prove that tuberculosis is caused by an invasion of the bacilli and is a parasitic disease mostly dependent on their growth and multiplication, one would have to isolate the bacilli from the body, grow them in pure cultures so that they are no longer dependent on any products of the disease, and finally transfer the isolated bacilli to animals and produce the same tubercular disease in the animal as with an infection of naturally obtained tuberculous material" (*36*).

Guinea pigs die within 4 to 6 weeks of inoculation with living tubercle bacilli. If the bacilli are inoculated, for example, in the left groin subcutaneously, in about 10 to 14 days an ulcerative nodule develops at the inoculation site. The ulcer remains open until the death of the animal. When necropsy is performed at about 6 weeks after inoculation, it can be observed that the lymphatic nodes draining the inoculation site and the lumboaortic nodes and the tracheobronchial nodes are hypertrophied; a section of these nodes shows that they are filled with a mass of necrotic material with the appearance of cheese. Because of its appearance, the necrotic tissue is known as caseum (the Greek word for cheese). Small, nodular, grayish-yellow lesions are apparent in the lungs, the liver, and the spleen. The spleen is greatly enlarged (splenomegaly).

Histologically, two types of lesions can be observed. The exudative lesion is an acute or subacute nonspecific inflammatory reaction characterized by the accumulation of fluid and of polymorphonuclear cells. The productive reaction is characterized by small microscopic granulomatous lesions known as follicles. These constitute the elementary tuberculosis lesions and are characterized by a central area of coagulation necrosis surrounded by layers of epithelioid and mononuclear cells. Amidst these cells, large ones containing many peripheral nuclei are observed. These giant cells are called giant cells of Langhans (Fig. 1). Tuberculosis progresses by contiguity and fusion of several follicles, which results in a larger and macroscopic lesion or tubercle. Outside the multiple layers of epithelioid cells there are proliferating fibroblasts and fibrotic tissue.

The productive lesion appears to evolve from the exudative lesion (*11*). Thus, during the course of the infection the character of the cellular response of the host changes. Koch found that tuberculous animals and noninfected animals react differently when they are superinfected with living tubercle bacilli. If after about 3 weeks of the primary infection the guinea pig is reinoculated intracutaneously, a nodule develops in about 2 days, and the skin over the nodule soon ulcerates. However, this ulcer heals quickly and the bacilli fail to spread. This phenomenon is known as the Koch phenomenon. Likewise, an inflammatory reaction can be elicited by inoculating dead bacilli, extracts of bacilli, subcellular fractions, or culture filtrates. The capacity of the tuberculosis host to circumscribe superinfection is indicative of acquired immunity, and the reaction of the infected host to culture filtrates indicates the development in the host of immunological changes of an allergic nature known as the tuberculin reaction.

TRANSMISSION AND PATHOGENESIS OF TUBERCULOSIS IN MAN

Tuberculosis is essentially an airborne disease. The bacilli are spread in the atmosphere in droplets expelled by patients with cavitary tuberculosis of the lung when they talk, cough, or sing, in increasing order of importance (*44*), as it is shown in Table 2. Thus, man is the chief reservoir of *M. tuberculosis* in nature. Wells, Riley, and their associates (*66, 88, 89*) demonstrated that the droplets that dry while settling to a size of about 2 to 3 microns are the main vehicles for transmission of the tubercle bacilli (Fig. 2). Each droplet nucleus carries very few bacilli (1 to 10), and it was demonstrated that the number of droplet nuclei initiating the infection approaches one. Furthermore, the probability of infection by one, two, three, or more droplet nuclei follows the Poisson series. These observations lead to three important considerations. One is that the probability of an individual to become infected is a function of the number of droplet nuclei containing bacilli that a patient in his vicinity produces, which in turn is a function of the concentration of bacilli in his secretions and of the severity of his cough. The second consideration is that ventilation (the number of air changes per unit time in a confined space) is an essential element in the control of tuberculosis infection. Finally, a third consideration is the probability that tuberculosis infections will be transmitted is usually small even though a single bacillus suffices to initiate the infection.

Generally the airborne particle is deposited in an alveolus in the well-ventilated middle or lower lobe of the right lung, just beneath the pleura. At the site of the implantation a nonspecific exudative inflammatory reaction occurs. The bacilli are phagocytosed by the fixed mononuclear cells of the alveolar duct (macrophages) in which they multiply. The bacillary multiplication eventually leads to the bursting and death of the cell. The freed bacilli are taken by other macrophages, and the process repeats itself. The exudative reaction becomes more extensive and caseating necrosis appears at its center. Thus, the early stage of the tuberculosis infection occurs in the alveolus (alveolitis). Tubercle formation begins at the periphery of the lesion and consists in the accumulation of layers of epithelioid

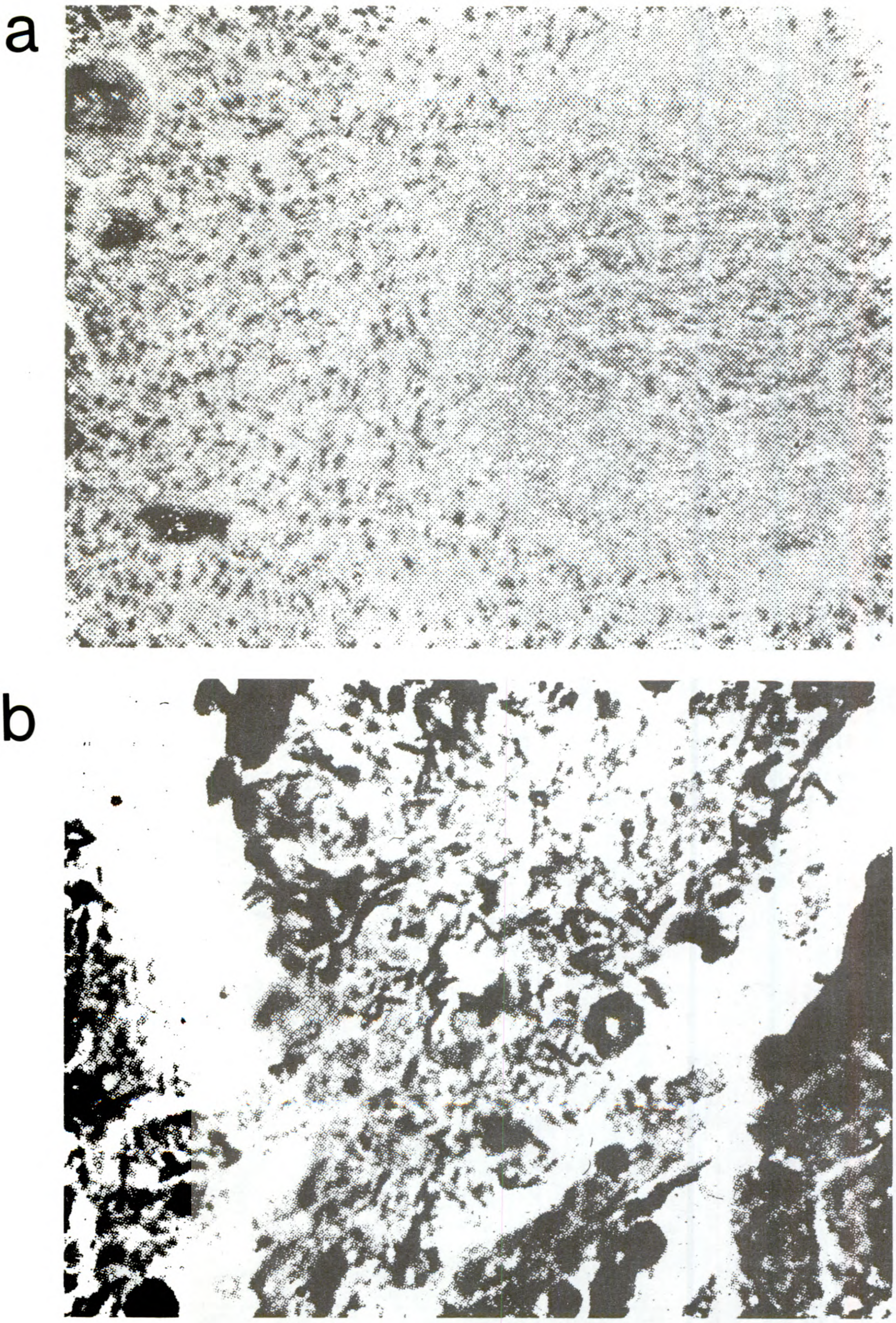
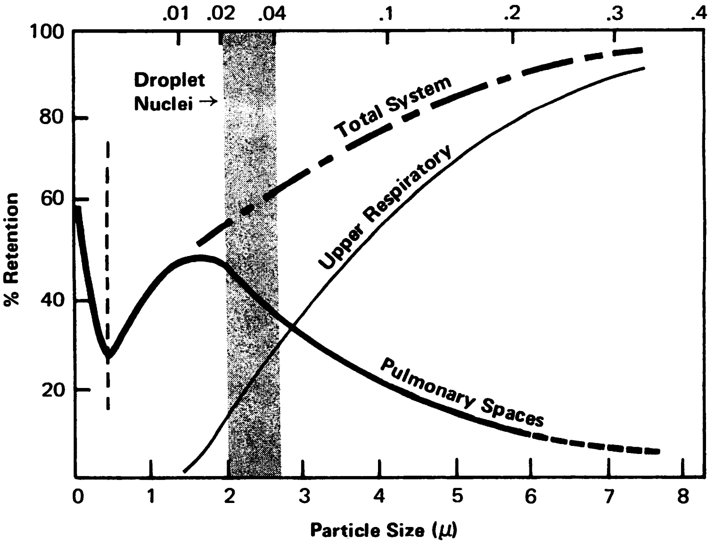


FIGURE 1. Section of tuberculous lung. A. – Tubercle: note the central area of necrosis surrounded by layers of epithelioid cells, and the giant cells. B. – Tubercle bacilli in the tuberculous lesion (original).

and giant cells. The bacilli spread from this primary focus of infection to the regional lymph nodes (tracheobronchial nodes) by way of the lymphatic vessels. They multiply in the nodes that become swollen due to inflammation and central caseating necrosis (lymphogenous dissemination). After reaching the efferent lymphatics, the bacteria eventually spread to the

TABLE 2. Percentage of Droplets of Stated Diameter
[adapted from Loudon and Roberts, *44*]

Calculated Airborne Diameter (μ)	Coughing	Talking	Singing
0.0–2.9	26.0	4.4	34.1
3.0–5.8	21.6	2.0	1.1
5.9–8.7	1.3		0.5
8.8–11.2	0.7	1.8	3.0
11.3–26.0	4.0	5.5	5.2
26.1–55.5	13.4	14.5	3.4
55.6–85.0	12.4	23.7	9.0
85.1–114.4	6.7	15.6	10.8
114.5–143.9	4.2	12.6	3.4
144.0–173.4	2.5	5.0	4.5
173.5–202.9	1.2	2.8	4.8
203.0–232.4	0.9	2.8	3.8
232.5–261.9	0.8	1.3	2.6
262.0–291.4	0.5	1.1	3.0
291.5–350.3	1.1	3.0	2.9
350.4–438.8	0.1	1.7	2.7
438.9–586.2	1.1	0.9	1.8
586.3–733.7	0.4	0.5	0.4
733.8–881.1	0.3	0.2	
881.2–1,028.5	0.1		
1,028.6–1,176.0	0.1		0.4
1,176.1–1,470.8	0.4	0.1	
>1,470.8	0.1	0.3	2.6



thoracic duct and the blood stream and are then carried to every tissue or organ in the body (hematogenous dissemination). The localization of the bacilli in the various organs appears to be conditioned by mechanical or anatomical factors rather than to a preferential environment (*65*). Within 4 to 12 weeks after infection the host becomes tuberculin positive. In the tuberculin positive host the lymphogenous dissemination stops, and most of the bacilli that become implanted in the organ die or remain dormant.

In most instances a positive tuberculin reaction is the only evidence of infection with the tubercle bacilli; in others, the healed lesion of the primary complex (pulmonary lesion and/or the regional lymph nodes) becomes calcified and can be detected by X-rays. In the U. S. in 1975 there are some 16,000,000 people that suffered a primary infection in the past, and most have not even realized that they were infected.

In some individuals, the primary infection progresses into disease. In children the most frequent forms of clinical primary tuberculosis are respiratory symptoms due to compression of a bronchus by the enlarged lymph nodes with resulting atelectasis; miliary tuberculosis; and meningitis. In the adult, primary tuberculosis disease may manifest itself as a minor clinical condition with parenchymal lung infiltration, as a more apparent pleuritic effusion, as pericarditis or peritonitis, or as chronic cavitary tuberculosis. Miliary tuberculosis in the young adult occurs rarely.

Years after the primary infection, a quiescent lesion in the lungs, kidneys, bones, brain, etc. may, for reasons still unclear, reactivate and cause clinical symptoms to emerge. Usually, tuberculosis in the adult is circumscribed to one organ, most often the lungs.

If pulmonary tuberculosis in the adult is the consequence of reactivation of an old primary lesion (reactivation tuberculosis), exogenous reinfection by superinfecting tubercle bacilli may also be a mechanism of acquiring pulmonary tuberculosis. The relative frequency of the two mechanisms (reactivation of an old lesion and exogenous reinfection) is unknown but may be related to the prevalent epidemiologic circumstances (*10*). In countries like the U. S., in which the morbidity for tuberculosis is low (about 14 per 100,000 inhabitants) and the control of tuberculosis is good, most cases are the result of the reactivation of old lesions located in the apex of the lungs. These lesions (Simon foci) are caused by the implantation of the bacilli during the systemic hematogenous phase of the primary infection.

Whether it is due to reactivation or superinfection, postprimary tuberculosis of the lung begins as a necrotic lesion usually of the apex, sometimes surrounded by extensive nonspecific inflammation. The necrotic lesion increases by contiguity, and when it reaches a bronchus the caseum material is discharged into it. The lung lesion is thus converted into a cavity, and the bacilli may now disseminate along the bronchial tree (bronchogenic dissemination), and other lesions may develop.

When the patient comes to seek medical advice, the destruction of the tissues is frequently already extensive, and most patients will eliminate tubercle bacilli that can be demonstrated in stained sputum smears. Radiographic films taken at this stage of initial tuberculosis show that the disease is in an advanced stage in more than 80% of the cases.

BACTERIOLOGIC DIAGNOSIS OF TUBERCULOSIS

The microscopic observation of acid-fast bacilli in stained smears of clinical secretions is not sufficient evidence for a diagnosis of tuberculosis. A correct bacteriologic diagnosis of tuberculosis requires the isolation of the acid-fact bacilli in pure culture and their identification as *M. tuberculosis*.

Most clinical specimens are contaminated by microorganisms that multiply faster than the tubercle bacilli. Hence, if they are not eliminated from the specimen, the slow-growing tubercle bacilli will be overgrown. Since the most frequent form of tuberculosis is pulmonary, sputum is naturally the kind of material most often handled in diagnosis. The sputum is a highly viscous material composed of the secretions of the glandular cells lining the bronchial mucosa, desquamating cells, inflammatory exudate, and necrotic caseous tissue. The successful isolation of the tubercle bacilli requires the liquefaction of the organic mass surrounding the bacilli (a process generally referred to as digestion or homogenization) and the killing of the undesirable contaminating organisms (a process generally referred to as decontamination).

The digestion-decontamination procedures in use nowadays are used more judiciously than before, but no one method is entirely harmless to the tubercle bacilli (Fig. 3). After digestion and decontamination,* the bacilli are concentrated to increase the chances of isolation, usually by centrifugation at 1,800 to 2,400 x g for 15 minutes.

The specimen treated as outlined above is inoculated onto the surface of solid media. The use of at least two media of distinct composition, for example the Lowenstein-Jensen medium and the Middlebrook and Cohn 7H10 medium, is recommended. The inoculated media are incubated at 36-37°C in an atmosphere of 5 to 10% CO_2 for at least 1 week with

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*For detailed description of these processes, see pages 154-157.

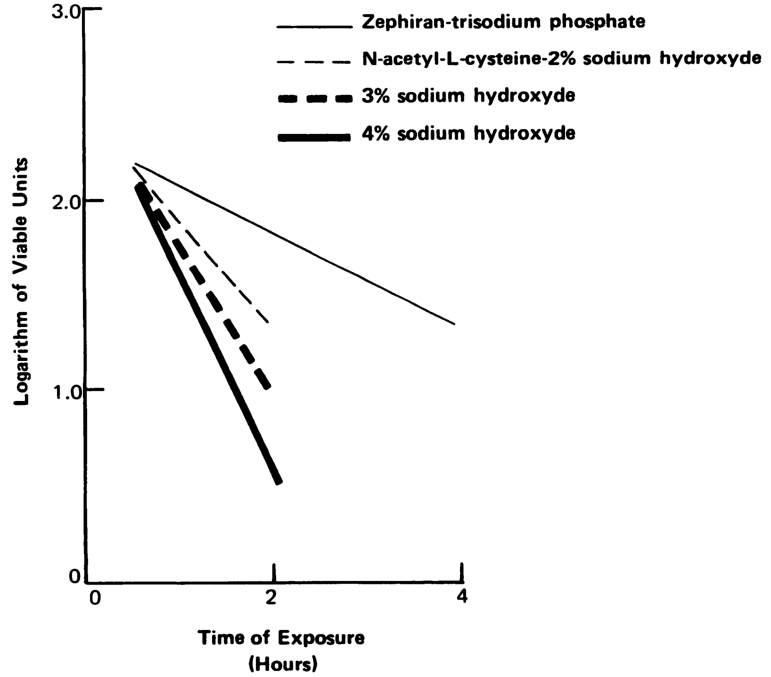

FIGURE 3. Death rates of *M. tuberculosis* in sputum disgested by four procedures (adapted from Krasnow, and Wayne, *37*).

the caps of the tube loose (Fig. 4). After 1 week of incubation, the caps are tightened, and incubation is continued for an additional 5 to 7 weeks. A culture must not be reported as negative before a minimum of 6 weeks' incubation.

Because of the nature of the specimen and the digestion-decontamination procedures, a certain frequency of contaminated cultures and culture failures is to be expected. Factors pertaining to the collection and handling of the specimens also contribute to the success in culture. An early morning specimen is preferable to pooled sputa, because it is less frequently contaminated and more often yields positive cultures (*35*). Another factor of importance is the time that elapses between the collection and processing of the specimens, which should not exceed 3 to 4 days.

An acceptable range of contamination is 1% to 3%. Whether the number of culture failures is too high may best be assessed by estimating the frequency of positive smears — negative cultures; this figure should be less than 1% in initial cases before chemotherapy is instituted; during chemotherapy the frequency of failures may rise to 7% or more. To avoid undue delays in diagnosis caused either by contamination or culture failure, three specimens should be collected on successive days.

As a procedure in the diagnosis of tuberculosis, routine guinea-pig inoculations are no longer justifiable for a number of reasons. First, and most important the proper use of culture methods is as sensitive or more sensitive than guinea-pig inoculation (Table 3); second, choice of the correct treatment of tuberculosis may be dependent upon the determination of the sensitivity of the infecting organisms to the chemotherapeutic agents; third, with this procedure other mycobacterioses of no less clinical and public health importance are not diagnosed; and, finally guinea-pig inoculation is more expensive.

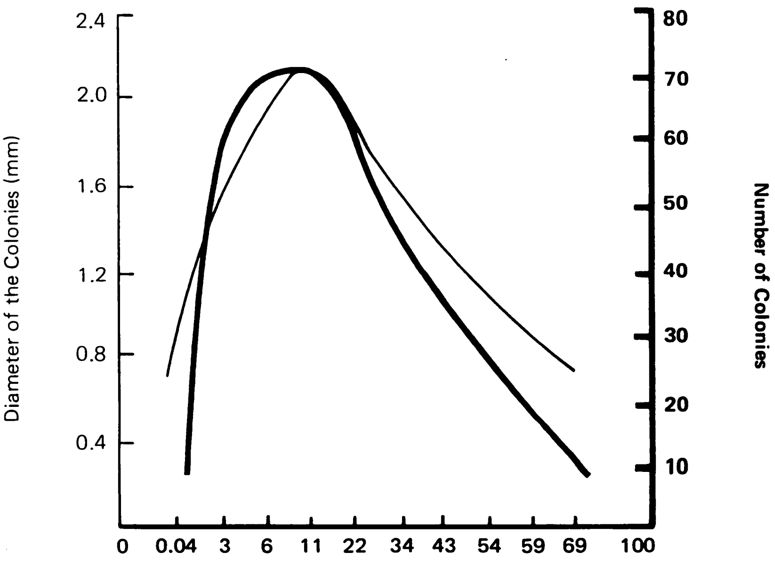

FIGURE 4. Effect of CO_2 on the growth (colony size and number of colonies) of *M. tuberculosis* on primary isolation from sputum (modified from Beam and Kubica, *1a*).

TABLE 3. Comparison of Guinea Pig Inoculation and Cultivation of *M. tuberculosis* from Clinical Specimens [adapted from Darzins, *20*]

Author	No. of Specimens Examined	Decontaminant	Frequency (%) of Positive Results		
			Medium		
			Petragnani	Lowenstein or L-J	Guinea Pig
Holm and Lester	20,090	H_2SO_4		16.9	14.5
Beller	212	HCl	77.8		72.2
Robinson and Dunn	209	NaOH		22.5	24.4
Sula	2,562	HCl		16.6	16.2
Abbott					
1. Sanatorium specimens	1,260	NaOH, Na_3PO_4		89.4	87.1
2. Field specimens	299	NaOH, Na_3PO_4		73.2	79.2

THE TUBERCLE BACILLI

•Morphology

When observed in stained smears of the pathological secretions, the tubercle bacilli are slightly bent rods, 2 to 4μ long and 0.2 to 0.5μ wide; they are evenly stained or are beaded and granular. The bacteria may grow separately or in small clumps. In laboratory media, the length of the bacilli varies with the growth conditions. On solid or in liquid media they tend to lie parallel to each and form long threads or "cords" (Fig. 5).

•Growth

The tubercle bacilli can be grown on solid or in liquid media. They are strict aerobes and grow better at pH 6.5 to 6.8. They are prototrophic, and their nutritional requirements are simple. Glycerol is a better source of carbon and energy than glucose. Although the organism is a prototroph, its isolation from pathologic materials requires the use of complex media. The reason for the higher nutritional needs of the bacilli grown *in vivo* is unknown. The growth rate and the cell yield are greatly enhanced by increasing the CO_2 tension in the atmosphere to 5-10% (*21,57*) (see Fig. 4).

The media used to cultivate the tubercle bacilli may be classified as complex natural, semi-synthetic, and chemically defined. Most complex natural media are egg based, and the most widely used one is the Lowenstein-Jensen medium, of which there are several modifications. Examples of semi-synthetic and chemically defined media are Long, Proskauer and Beck, Merril, Youmans, Dubos and Davis, Middlebrook and Cohn, and Goldman. Compositions of these media are represented in Table 1, Part 1, Chapter 3.

•Resistance to Physical and Chemical Agents

The tubercle bacilli are no more resistant to moist heat than other bacteria. However, next to *Coxiella burnetti* they are the most resistant of the nonsporeforming pathogenic bacteria. For this reason, the temperature and time (143°F, 30 minutes) used in pasteurization techniques, were increased enough to kill the tubercle bacilli (Fig. 6). The discovery that *C. burnetti*, a Rickettsia that causes Q Fever, can be transmitted by milk and is more resistant to heat than the tubercle bacilli led to a 2°F increase in the required temperature.

Milk is pasteurized to eliminate pathogenic bacteria. One process consists of holding the milk at 145°F (63°C) for 30 minutes and then refrigerating it. This is the Low Temperature Holding (LTH) method. Another method is the High Temperature-Short Time (HTST) or flash method, whereby the milk is heated rapidly to 71.6 to 80°C in three layers between metal plates, is held at this temperature for 15 to 20 seconds, and then is cooled. In neither case is the milk sterilized. Because fresh milk normally contains a phosphatase slightly less heat sensitive than the tubercle tacilli (Fig. 6), pasteurized milk that gives negative phosphatase test results is considered adequately pasteurized in practice.

In dried sputum the bacilli may remain alive for over 8 weeks if protected from direct sunlight; when exposed to sunlight they are killed within 20 to 30 hours (36). Droplet nuclei may remain infectious for 8 to 10 days. The bacilli are very sensitive to ultraviolet light irradiation of a wavelength around 2537Å, which is the predominant wavelength of the rays emitted by the usual low pressure, mercury vapor germicidal lamps. Quantitative studies on the rate of killing by UV irradiation showed that the tubercle bacilli are no more resistant than other nonsporeforming bacteria. (See Part 1, Chapter 4).

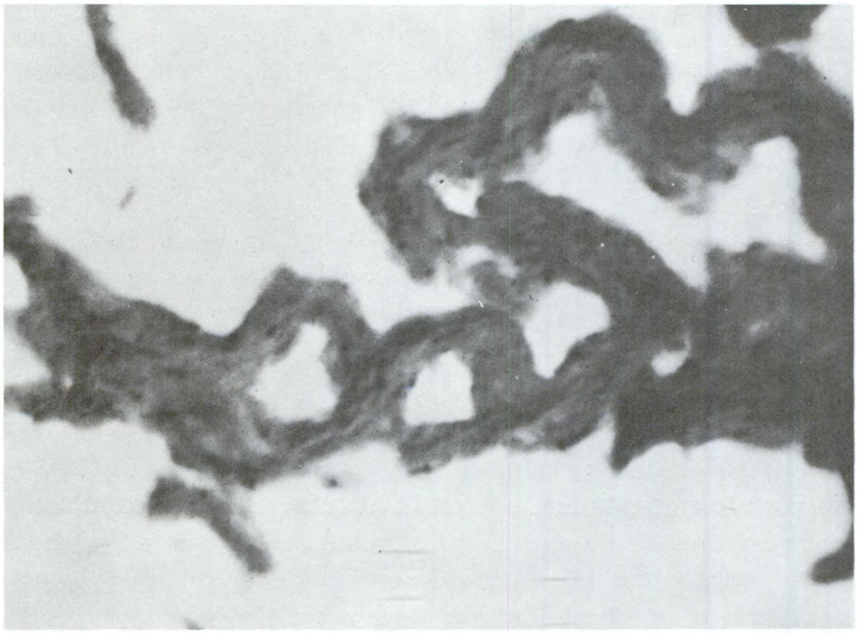

FIGURE 5. Serpentine pattern of growth ("cording") in *M. tuberculosis* (courtesy of R. Smithwick).

The tubercle bacilli and the other mycobacteria have relatively high resistance to the effects of exposure to acids or alkalis, quaternary ammonium salts, and other common disinfectants. This high resistance is used to advantage in isolating them from pathological specimens. However, it must be emphasized that they are killed by those agents if exposed for too long (*37,38*). A 5% solution of phenol kills the tubercle bacilli suspended in distilled water within a few minutes, but 24 hours is necessary to sterilize sputum. A 5% solution of the common household bleach sodium hypochlorite kills the tubercle bacilli in sputum in

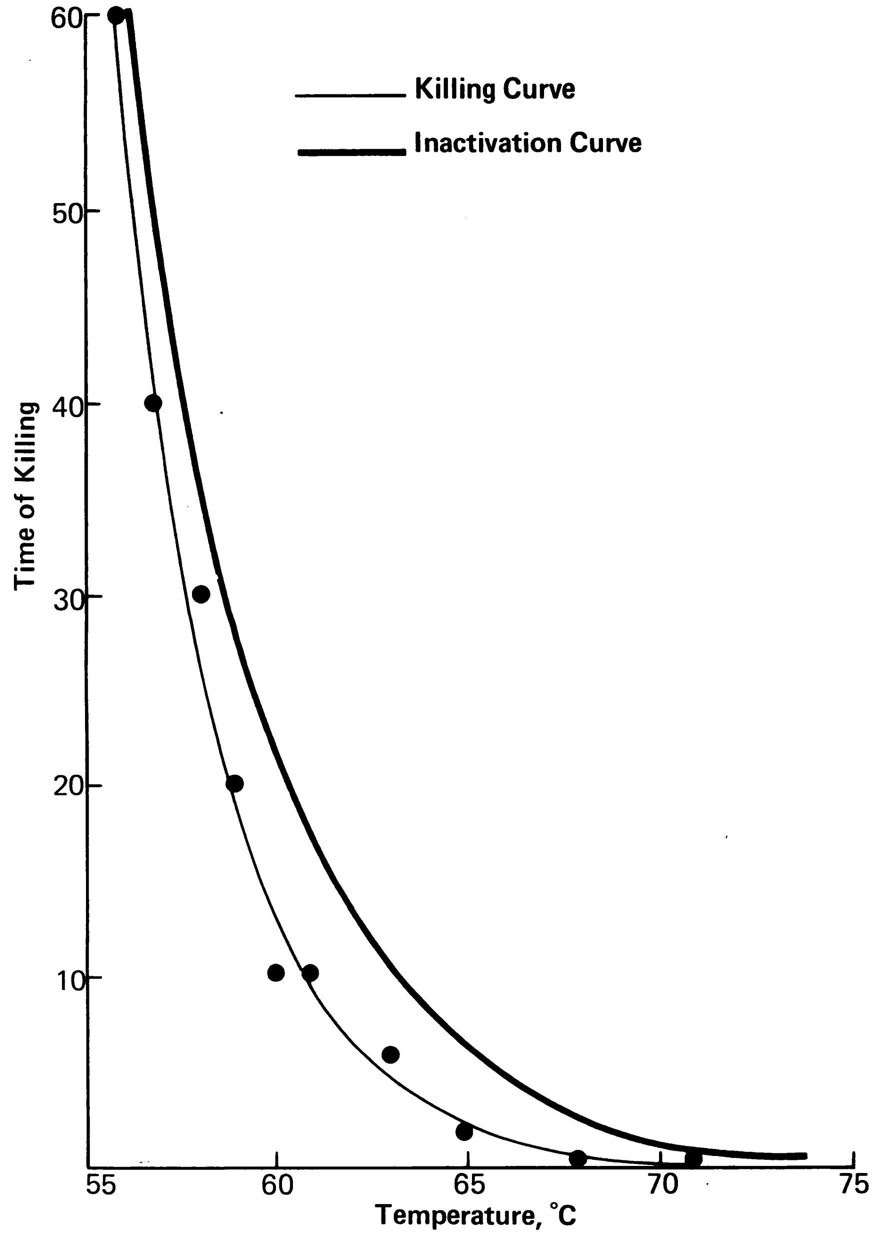

FIGURE 6. Temperature-time relationships in the killing of *M. bovis* in milk, and inactivation curve of milk phosphatase. The killing curve is a plot of data from North and Park (*56*); the inactivation curve for phosphatase is hypothetic.

about 15 minutes (*59*). Although a number of disinfectant agents are used in laboratories, most of them are of limited value. To be useful in the laboratory, a disinfectant must: (1) kill the tubercle bacilli rapidly; (2) act in the presence of organic material (sputum, tissue, etc.); (3) be relatively nontoxic to the user; and (4) remain stable for prolonged periods of time.

PATHOGENICITY AND VIRULENCE

The guinea pig is the most sensitive of all the experimental animals. The extension of the disease and its severity and outcome depend on the number of bacilli inoculated, the route of infection and/or the properties of the bacteria themselves that are described by the word "virulence." Strains of the tubercle bacilli inoculated in guinea pigs under standard conditions may elicit disease to a different extent, and these differences are measurable and referred to as degrees of virulence. Thus, some strains will cause a local lesion with virtually no spreading even if large numbers of bacilli are injected, whereas a single bacillus from other strains may cause progressive and fatal disease. The strains that exhibit low virulence are known as *attenuated*. Strains of low virulence are easily obtained by successive transfers *in vitro* (*23*). This is due to the selection of attenuated mutants, but the selective process may be counteracted to favor the progressive establishment of the few virulent cells remaining, or of virulent revertants by successive transfers of the attenuated strain through guinea pigs. (See Part 1, Chapter 4: Genetics of the Mycobacteria).

•Bacterial Determinants of Pathogenicity and Virulence

Middlebrook, Dubos and Pierce (*51*) recognized that the variations in the appearance of the colonies of the tubercle bacilli were related to their virulence in the guinea pig. Thus, they showed that strains that form microscopic serpentine cords are usually virulent, and those eugonic strains which grow in a nonoriented fashion are usually attenuated. A toxic glycolipid, or "cord factor," was extracted from the vi$^+$ cells with petroleum ether. Most of the cord factor is present in Anderson's Wax C (Part 1, Chapter 2). The extraction of the "cord factor" does not kill the cells, but disrupts the bacterial cords, and according to Bloch, the extracted bacilli lose the ability to cause progressive disease in guinea pigs. The compound causes pulmonary hemorrhagic lesions in mice that may result in death. However, these pathologic reactions are manifest only after a series of injections, and hence they may be due to hypersensitivity rather than to a direct toxic effect. The substance inhibits the migration of leukocytes *in vitro* and is said to enhance latent infections in a way similar to cortisone (*2, 3, 4*).

Strains that do "cord" usually bind the dye neutral red (*22*) in an alkaline aqueous environment. This property constitutes the basis for a test designed to differentiate vi$^+$ from vi$^-$ strains (neutral red test), but the test is no longer in use because of the many exceptions to the correlation between a positive neutral red test and virulence. Sulfolipids extracted by hexane from virulent strains were found to become bright red when shaken in a dilute aqueous solution of neutral red hydrochloride (*74*). These were fractionated (*27,28*), and one was identified as a complex, 2, 3, 6, 6′ tetraacyl-1-trehalose - 2′ - sulfate. The fatty acids esterifying the trehalose - 2′ - sulfate moiety were characterized as palmitic acid, heptamethyl - triacantonoic acid, and 17 - hydroxy - octamethyl - dotriacantonoic acid (*29*).

•Pathogenicity and Virulence (isoniazid-resistant mutants)

The observation of Grunberg (*30*) and Steenken and Wolinsky (*78a*), that tubercle bacilli resistant to the chemotheropeutic agent isoniazid were of low virulence for the guinea pig stimulated a considerable amount of investigation. Inh^{r} is caused by spontaneous mutations that occur at very low frequencies (see Part 1, Chapter 4). Middlebrook (*52*) described two types of Inh^{r} mutants: one-step mutants resistant to low concentrations of INH were usually cat^{+} and vi^{+}, whereas one-step mutants resistant to high concentrations (10 μg/ml or more) were cat^{-} and vi^{-}. His observations were confirmed by a large number of investigators.

Inh^{r} mutants are selected during therapy with INH. Only seldom, if ever, is an isolated population composed exclusively of Inh^{r} cells. When an Inh^{r} cat^{-} freshly isolated population is inoculated into guinea pigs and these are sacrificed at various intervals, the local lesion and the regional lymph node involvement appear to progress as usual until about the fourth week. From then on, the disease spontaneously regresses (regressive tuberculosis of Karlson and Ikemi, *33*), but there is a relapse much later with the features of progressive and fatal tuberculosis. The bacilli isolated at necropsy differ from the inoculated population in that they are Inh^{s}. This phenomenon is caused by the presence in the original population of a few Inh^{s} cat^{+} vi^{+} bacilli. Whether the progressive phase of the infection occurs earlier or later depends upon the ratio of vi^{-} to vi^{+} cells in the inoculum, but it also depends on the development of specific acquired resistance elicited by the initial infection (*94*). When artificial populations are constructed by mixing Inh^{s} and Inh^{r} cells in various proportions, 2% Inh^{s} bacteria is sufficient for only sensitive organisms to be isolated from the tissues of the animals that died from progressive tuberculosis (*33*).

The above phenomena are further examples of the selective mechanisms described in an earlier chapter. To a certain extent the conflicting data in the literature regarding the relationships between virulence, isoniazid resistance, and other properties of the tubercle bacilli (cording, neutral red binding, catalase activity, and others) are due to the failure of the investigator to recognize the heterogeneity of the bacterial population he was dealing with. Indeed, better correlation is obtained in populations that are selected through successive transfers in media containing high concentrartions of the drug (*20a*). However, in spite of the extensive work done, a full analysis with the methods of population genetics is still lacking. For example, it is still unclear whether the alterations associated with Inh^{r} are caused by a mutation that affects a series of genes or whether they are independent mutations caused by isoniazid, itself a mutagen (*25*).

In man, Inh^{r} mutants can cause infection and eventually progressive disease. A recent well-documented epidemic caused by Inh^{r} mutants (*79,80*) indicates that patients excreting these bacteria must not be lightly regarded.

Also, the finding that some initial cases are infected with Inh^{r} tubercle bacilli (see Primary drug-resistance, described below) shows that these mutants remain virulent for humans.

BACTERIOLOGICAL BASIS OF THE CHEMOTHERAPY OF TUBERCULOSIS. DRUG-SENSITIVITY TESTS

In 1947, M. Pyle (*62*) showed that the ratio of the Str^{r} to Str^{s} bacteria in strains isolated from the secretions of patients during chemotherapy increased with time. This observation originated the very important notion that unresponsiveness to therapy in tuberculosis may be apparent before the direct selective process is completed. The *in vivo* selection of Inh^r mutants also proceeds very rapidly during monotherapy. Thus, about 64% of the cultures isolated from patients treated for 3 months and 93% of those treated for 6 months were found resistant by the Medical Research Council (England) Isoniazid Trial No. 3 (*49*). Since selection can occur only if bacterial variation (mutation) occurred before exposure to the drug, the outcome of the therapy of tuberculosis depends upon the size of the mutant clones that happen to be present at the time therapy is initiated.

Bacterial mutations occur at very low but measurable frequencies (Part 1, Chapter 4), and the probability of two independent mutations occurring in a single bacterium is even less. Mathematically, the probability of two independent events occurring simultaneously is given by the product of the probabilities of occurrence of each one. Since the mutations are independent events and the forward mutation rates to various drugs are known, the probability of finding a double mutant in a population may be calculated. For example, the probability of finding a double mutant resistant to Inh and Str is about 7.6×10^{-16} (mutation rate for Inh = 2.56×10^{-8}; mutation rate for Str = 2.95×10^{-8}; probability of a double mutation = $(2.56 \times 10^{-8}$ $(2.95 \times 10^{-8}) = 7.552 \times 10^{-16}$ mutations per bacteria per generation). If the probability that a randomly chosen bacterium will become a double mutant within a generation time is 7.552×10^{-16}, this means that within populations of size 10^{16}, the average number of double mutants is $7.552 \times 10^{16} \times 10^{-16} = 7.552$. The probability that a population of size 10^{16} contains at least one double mutant is 0.99945. This does not mean that populations of sizes 10^5, 10^6 or 10^7 cannot have double mutants, although the probability of this event is certainly negligible. Therefore, the selection of mutants resistant to one drug (but not the mutations) can be avoided by simultaneously exposing the population to a second drug. The results that were obtained in large scale chemotherapy trials organized to evaluate different single and combined drug regimens clearly demonstrated that combined drug-therapy is: 1) more often conducive to bacteriological conversion; and 2) very effective in reducing the frequency of therapeutic failure caused by the selection of drug-resistant mutants. On the basis of these therapeutic trials, the antituberculosis drugs were classified as primary and secondary drugs. Primary drugs are those that must be preferred in treating initial cases, and the secondary drugs are reserved for the treatment of patients excreting bacteria resistant to the primary drugs.

The correct treatment of patients with tuberculosis requires the determination of the sensitivity of the infecting bacteria to the chemotherapeutic agents. Up until now the expression "drug-resistance" has been avoided in referring to bacterial populations. It has been used only to describe individuals within a population. Indeed, resistance is the ability of a bacterium to survive the exposure to a drug at a concentration that inhibits or kills the parental cells and to transfer this characteristic to its progeny. The progeny of a resistant bacterium (a mutant) constitutes a resistant population in which the proportion of drug resistant cells approaches 1.0 (100%). This genetic definition of a drug-resistant population does not serve a useful purpose in the treatment of tuberculosis because, as mentioned earlier, lack of response to therapy may be apparent before the bacterial population is resistant in the sense stated above. This fact underlines the difficulty in reaching an agreement about a definition of resistance in tuberculosis. However, criteria of resistance applicable to populations (strains) were reached by empirical means. All the criteria proposed and the methods adopted are based on the recognition that there is a proportion of mutants below which a strain may be safely classified as susceptible for medical purposes.

The procedures used to perform drug-sensitivity tests and the criteria adopted to interpret the laboratory data have changed throughout the years. This is the result of a better understanding of the factors that affect the testing in the laboratory and of the host-parasite relationships, which has led to development of two fundamental concepts: the "critical concentration" of the drugs in the media and the "critical proportion" of mutants.

The "critical concentration" of the drug is that concentration which inhibits the growth of the wild-type parental organisms but is not so high that the mutants are also inhibited. Two procedures can be used to obtain the necessary information. By far the most extensively used method is that of finding the minimal concentration of a drug required to inhibit strains isolated from untreated patients. A range of values is thus obtained, and the concentration that inhibited all or almost all of the strains is adopted in routine testing. An alternative procedure is to perform a fluctuation analysis. The critical concentration is that concentration that allows the drawing of a line between wild-type parental cells and their mutants. The two procedures are exemplified in Table 4.

The largest mutant clone that is usually obtained in fluctuation tests may be considered the highest proportion of mutants that accumulate as the result of spontaneous mutations. Certainly, the size of the mutant clone is large if the mutation occurred very early, and if in the fluctuation analysis a small culture was inoculated by chance with a mutant, the proportion of mutants is approximately 1.0 (100%). Although in theory the size of the mutant clone may range from 0 to 100%, in practice the largest mutant clones have a size of around 1 x 10^{-6}. Any proportion of mutants that is higher than the one calculated by genetic analysis is due to selection favoring the mutants. These values are obtained by bacteriological means alone. In contrast, the fundamental question of how much selection is compatible with therapeutic success (or critical proportion of mutants) cannot be answered by bacteriological means alone. The critical proportion for various drugs was established by Canetti, Rist and Grosset (*9*). Table 5 is an example of how the critical proportions are calculated. It summarizes the drug-sensitivity (ethambutol) patterns of strains of the tubercle bacilli isolated from 34 resected lung specimens: 17 specimens were from patients that were not treated with the drug being analyzed; 6 specimens were from patients that were treated for less than 3 months; 6 specimens were from patients treated for 3 to 6 months; and 5 specimens were from patients treated for at least 6 months. The proportion of ethambutol resistant cells in each of the strains was established by the proportion method, and then four possible criteria were considered: 1) strains containing 1% or more resistant cells at 1 μg/ml are to be classified resistant; 2) strains containing 10% or more resistant cells at 10 μg/ml are to be considered resistant; 3) strains containing 1% or more resistant cells at 2 μg/ml are to be considered resistant; and 4) strains containing 10% resistant cells at 2 μg/ml are to be considered resistant. Once data is so arranged, the problem consists of deciding which of the hypotheses fits best the expectations in terms of the clinical response and the kinetics of selection of the resistant bacteria. The first hypothesis is clearly unsatisfactory. Indeed, before any treatment 24% of the strains already would have been classified as resistant, which is in total disagreement with the results of therapeutic trials as well as with bacteriological data; the second, the third and the fourth criteria could be acceptable. However, the hypothesis that strains containing at least 1% resistant cells at 2.0 μg/ml are considered resistant is the best criterium, because it agrees with therapeutic trials and the selection that occurred progressed regularly as expected (increase in the proportion of resistant cells as a function of the duration of therapy). Canetti and his associates used this procedure to determine the critical proportion of

TABLE 4. Distribution of D-CS Resistant Mutants Among Identical and Independent Cultures of Tubercle Bacilli

Both procedures indicate that the critical concentration of D-CS is 20 g/ml. ▨
The mutation rate was calculated to be 0.9 x 10^{-10} mutations per bacterium per generation;
the highest proportion of mutants was 9.4 x 10^{-9}, or about 1 x 10^{-8}.

	Identical Cultures (fluctuation test)[1]							Independent Cultures[2]				
	2.5	5.0	10.0	20.0	30.0	40.0	50.0	Inoculum	10.0	20.0	30.0	50.0
1	2+	182	0	0	0	0	0	200	0	0	0	0
2	2+	60	94	0	0	0	0	400	0	0	0	0
3	107	6	1	0	0	0	0	2,600	260	0	0	0
4	2+	2+	1+	0	0	0	0	200	0	0	0	0
5	15	2	0	0	0	0	0	300	8	0	0	0
6	1+	1+	95	8	0	0	0	100	0	0	0	0
7	2+	2+	2	0	0	0	0	50	14	0	0	0
8								100	0	0	0	0
9	2+	2+	2+	1	0	0	0	50	50	0	0	0
10	2+	2+	5	0	0	0	0	30,000	6,000*	0	0	0
11	1+	1+	16	0	0	0	0	500	250	0	0	0
12								1,000	1,000*	0	0	0
13	1+	2+	112	0	0	0	0	500	2	0	0	0
14	1+	1+	1+	0	0	0	0	50	50	0	0	0
15	2+	2+	41	0	0	0	0	50,000	50,000*	0	0	0
16	2+	2+	2+	33	0	0	0	10,000	10,000*	0	0	0
17	2+	2+	2+	1	0	0	0	20,000	20,000*	0	0	0
18	2+	2+	2+	2	0	0	0	1+	1+	0	0	0
19	2+	2+	2+	0	0	0	0	30,000	30,000*	15,000*	300	0
20	2+	2+	2+	7	0	0	0	100	0	0	0	0
21								5,000	0	0	0	0
22								50	0	0	0	0
23								1,000	0	0	0	0
24								50	50	0	0	0
25								1,000	1,000*	0	0	0
26								50	0	0	0	0
27								300	0	0	0	0
28								1,000	0	0	0	0
29								1+	1+	±(~50%*)	0	0
30								200	0	0	0	0
31								50	1	0	0	0
32								100	0	0	0	0
33								50	20	0	0	0
34								1,500	2	0	0	0
35								800	200	0	0	0
36								100	100	0	0	0
37								100	100	0	0	0
38								200	50	0	0	0
39								50	6	0	0	0
40								200	8	0	0	0

[1] Inoculum size: 3.5 x 10^9 cells.

[2] Inoculum size obtained by diluting cultures on 7H-9 medium.

*Calculated number of colonies.

resistant cells to every drug now in use in the treatment of tuberculosis (Table 6). The critical proportion, in contrast to the critical concentration, is independent of the testing conditions. Thus, as shown in Table 6, the same critical proportion of mutants is used to classify strains as sensitive or resistant in both Lowenstein–Jensen medium and the Middlebrook and Cohn's 7H10 medium at the respective critical concentrations.

TABLE 5. Determination of a Criterium of Resistance: Ethambutol
(Lowenstein-Jensen medium, drug incorporated before inspissation)

Duration of Administration of Ethambutol (Months)	Total No. of Culture Positive Lung Specimens	Criterium of Resistance											
		⩾1% at 1 mcg/ml			⩾10% at 1 mcg/ml			⩾1% at 2 mcg/ml[1]			10% at 2 mcg/ml		
		Sens.	Res.	% Res.	Sens.	Res.	% Res.	Sens.	Res.	% Res.	Res.	Res.	% Res.
0	17	13	4	24	17	0	0	17	0	0	17	0	0
<3	6	2	4	67	4	2	33	5	1	17	6	0	0
3-6	6	3	3	50	4	2	33	4	2	33	5	1	17
⩾6	5	0	5	100	1	4	80	2	3	60	4	1	20

[1] The criterium actually adopted is shaded.

NOTE: Confidence on the criterium: the maximal level of drug-resistant mutants growing at 2 mcg/ml in wild strains is 2.0×10^{-5} (reading at the 42nd day of incubation).

(Courtesy of Dr. George Canetti, Institut Pasteur, Paris.)

TABLE 6. Critical Concentrations of Drugs and Critical Proportions of Mutants of the Tubercle Bacilli

Drug	Critical Concentrations (µg/ml)		Critical Proportions[3] (µg/ml)
	Lowenstein-Jensen[1]	7H-10[2]	(%)
Isoniazid	0.2	0.2	1.0
Streptomycin	4.0	2.0	1.0
PAS	0.5	2.0	1.0
Kanamycin	20.0	5.0	10.0
Ethionamide	20.0	5.0	10.0
Viomycin	30.0	5.0	10.0
D-cycloserine	30.0	20.0	10.0
Pyrazinamide	100.0	50.0	10.0
Rifampin	40.0	1.0	1.0
Ethambutol	2.0	2.0	1.0*

[1] From Canetti et al (8). This data is being used with the permission of the Pan American Health Organization and the author.

[2] Data from CDC.

[3] According to Canetti et al (8).

*Canetti, personal communication.

PRIMARY DRUG RESISTANCE

Primary drug resistance is an epidemiological concept that refers to the demonstration of drug resistance of strains isolated from patients that have not received treatment. These are individuals who were initially infected by resistant bacilli excreted by a source case in which resistance has developed by virtue of therapy. Because tuberculosis infection is air-borne and infection is usually caused by small numbers of bacilli, the probability of infection with drug resistant bacilli must be a direct function of the proportion of drug-resistant bacteria excreted by the source case. A corollary to this is that the higher the prevalence of primary drug resistance, the worse the control and management of tuberculosis. Thus, primary drug resistance surveys are important epidemiological tools to estimate the quality of the tuberculosis programs in a city, country, or region.

By definition, primary drug resistance implies that the patients did not receive any antituberculosis drugs. Sometimes this is difficult to establish, and in such instances the expression initial drug resistance is used instead (drug resistance in patients that give no history of previous chemotherapy).

Surveys of primary drug resistance are important in planning large scale treatment programs and must be carried out in a central or reference laboratory by acceptable procedures. After a survey in a region, country, or city, a surveillance program of indefinite duration must be instituted. The surveillance program is required to determine whether the prevalence of primary drug resistance remains at an acceptable level, whether it decreases from a high level to an acceptable level soon after a tuberculosis control program was started, or whether within the framework of a good program there is a sudden and unexpected increase which requires corrective measures.

INDICATIONS OF DRUG-SUSCEPTIBILITY TESTING

In countries like the U. S. where the continuing primary drug resistance surveillance indicates that the majority of new cases of tuberculosis are infected with sensitive bacteria, treatment is initiated once tuberculosis is proven. Persistent productive cough, weight loss, anorexia, low fever and night sweats, although common to many illnesses, are symptoms that associated with: 1) a recent tuberculin conversion; 2) an X-ray examination showing suggestive or definite lung lesions; and 3) a finding of acid-fast bacilli in sputum smears constitute the basis of a presumptive diagnosis of tuberculosis. Although the specific diagnosis of tuberculosis requires the isolation and characterization of the causative agent, this prerequisite is not necessary to initiate treatment in most situations.

The main indications of drug-susceptibility testing in tuberculosis are: a) identification of primary drug resistance (PDR); and b) readjustments of therapeutic regimens.

•Primary Drug Resistance

The expression primary drug resistance, or PDR, refers to a patient who has never been treated and from whose secretions drug-resistant tubercle bacilli have been isolated. A PDR case is a patient who was initially infected with drug resistant bacilli. To identify these cases, drug-susceptibility studies must be performed with a strain isolated before chemotherapy is started.

Whether these studies are necessary in all initial cases or only in a sample of the total number of cases depends on epidemiological circumstances, as summarized below.

Figure 7 gives a generalized view of the events that occur during therapy in 85% or more of the smear-positive initial cases of tuberculosis. Most patients are in an advanced stage when they first seek medical attention, and microscopic examination of the sputum usually demonstrates the presence of acid-fast bacilli. Before chemotherapy is initiated, three successive early morning specimens should be collected for (1) microscopic examination of acid-fast stained smears and (2) isolation and identification of tubercle bacilli. Because one must recognize that isolation may fail (as is the case in less than 1% of all initial cases) or the cultures may be ruined by contamination (an average of 3%), a single specimen is not satisfactory, and experience has shown that more than three to five specimens do not appreciably increase chances for a specific diagnosis.

One of the initial positive cultures may be used to perform a drug-susceptibility test. So long as there is no evidence of therapeutic unresponsiveness, or therapeutic failure, the test need not be repeated.

The U. S. Public Health Service (PHS) has maintained a nationwide surveillance of PDR for over 20 years. The surveillance showed that PDR for isoniazid, streptomycin, and p-amino salicylic acid has remained constant (about 4%, 8%, and 5%, respectively). The data for ethambutol and rifampin indicate that PDR is low in respect to these drugs (2% and 0.6%, respectively). The PDR surveillance gives some information regarding the overall situation, but it may not reflect circumstances as they occur in every community. Thus, we may conceive that the incidence of PDR may be higher than the average in a large city where management and control of patients is difficult. In addition, in a community where treatment practices are poor and, therefore, the frequency of therapeutic failure is high, one may expect a high incidence of PDR because the probability of infection with drug-resistant bacilli increases with the number of therapeutic failures in patients who can then act as sources of infection.

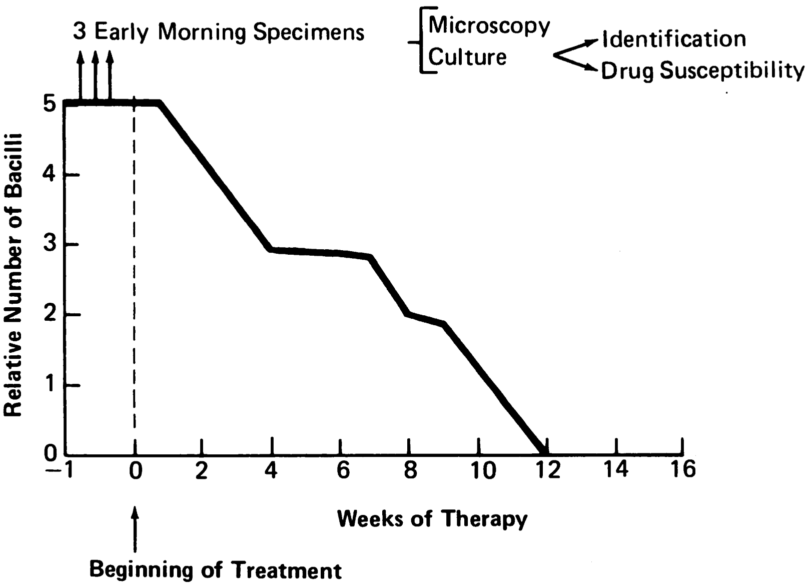

FIGURE 7. Generalized view of bacteriologic response (microscopy) during chemotherapy. Case of successful therapy.

A high incidence of PDR usually reflects deficiencies in either the treatment practices or the control of patients under treatment. In these communities, initial drug-susceptibility tests in all cases may be recommended and corrective measures must be instituted. On the other hand, where treatment practices are good and monitoring of patients is efficient, one may agree that initial drug susceptibility tests are needed in only a fraction of the new cases as a means of maintaining close surveillance of the epidemiologic situation.

•Readjustments of Therapeutic Regimens

The indications for drug-susceptibility testing in initial cases were described above. Figure 8 gives a generalized view of the events that may occur in cases of therapy failure. Failure may be caused by drug resistance, but if a patient discontinues treatment against advice, relapse will occur, and the bacilli may remain sensitive. In either situation, drug-susceptibility testing is necessary to determine what regimen is most suitable.

Even if chemotherapy is successful, drug resistant mutants are at a selective advantage. Thus, as the number of colonies on culture media declines during chemotherapy, some of these colonies may be composed of drug-resistant bacteria. Clearly, drug-susceptibility tests performed on fewer and fewer colonies may yield misleading results. Thus, drug tests should not be performed during the initial stages of therapy but only when there is evidence of therapeutic failure or in cases of relapse.

For example, if during uneventful treatment a culture yields a single colony, the chances are high that it is from one of the few resistant mutants still being excreted. A drug-susceptibility test will demonstrate just that, but the patient will be responding well. The obvious discrepancy does not necessarily mean that the test is useless, nor that failure is occurring and a change in therapy is warranted. It may mean, however, that the test was requested at the wrong time.

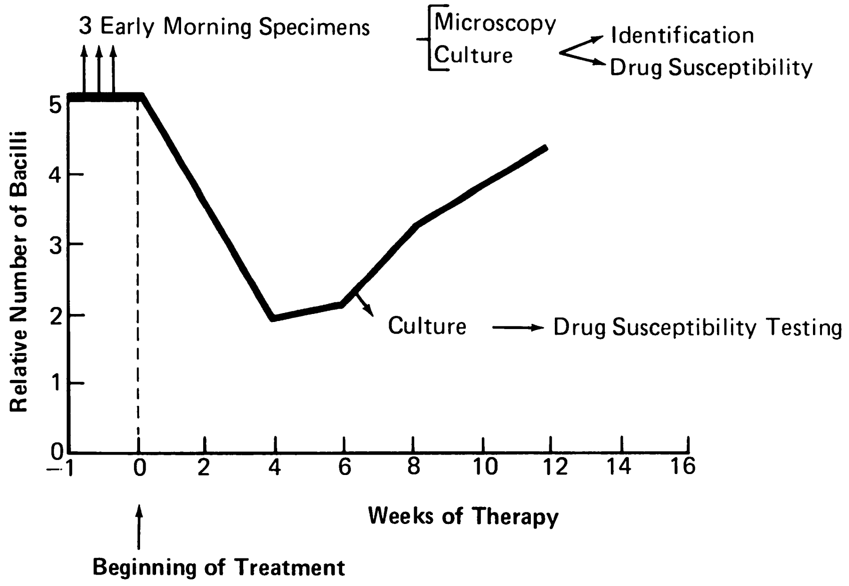

FIGURE 8. Generalized view of bacteriologic response (microscopy) during chemotherapy. Case of unsuccessful therapy.

CONTROL OF TUBERCULOSIS

In the U. S. the program for the control of tuberculosis in humans is based on the identification (case finding) and treatment of the diseased individual (stop the chain of transmission), the identification of the infected reservoir (tuberculin tests), and the elimination of sources of infection other than humans (cattle, milk, and milk products). Because the U. S. accepts large numbers of immigrants every year, tuberculosis control is extended to include reducing the danger of importing the disease, with the help of the U. S. Embassies overseas and the local health authorities.

The overall size of the tuberculosis problem can be measured by the following parameters: 1) tuberculin reactors; 2) new infections; 3) new active cases of tuberculosis/year; 4) number of people affected by tuberculosis/year; and 5) deaths by tuberculosis.

Each of these parameters measures a different aspect of the tuberculosis problem in the community. The tuberculin reactors are an index of the prevalence of infection with the tubercle bacilli. It is from this group that most tuberculosis cases emerge every year. In 1971, of the U. S. population of about 206,000,000, the total number of tuberculin reactors was estimated to be about 16,000,000 (Fig. 9). This group of people contributed 36,500 cases of tuberculosis out of the 39,500 known new cases that year. The remaining 3,000 cases were due to the progression of recent infections.

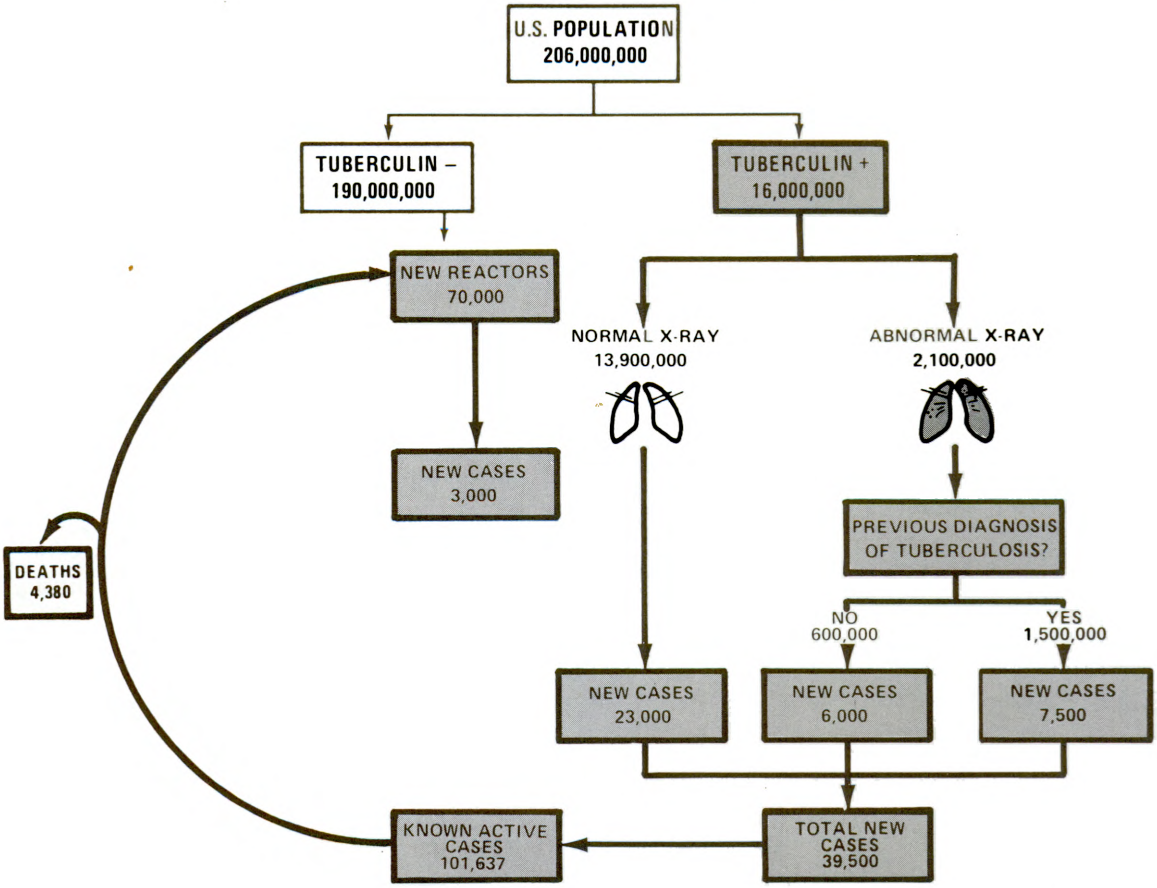

FIGURE 9. Tuberculosis in the U.S., 1971 (courtesy of Dr. P. Edwards, CDC).

X-ray data may be used to analyze the risk of contracting tuberculosis among the tuberculin reactors. It has been estimated that of the 16,000,000 reactors, 13,900,000 had normal X-rays and 2,100,000 has abnormal X-rays. Of the 2,100,000 with abnormal X-rays, 600,000 had no previous diagnosis of tuberculosis, whereas 1,500,000 have had tuberculosis sometime in the past. How much tuberculosis occurred in each of these groups is shown in Figure 9. The diagnosis shows that the risk of having tuberculosis disease is higher in the tuberculin reactor-abnormal X-ray group (about 645 cases per 100,000) than in the tuberculin reactors with normal X-rays (165 cases per 100,000).

The overall number of new cases in 1971 was 39,500 (14.5 per 100,000 inhabitants). This includes new cases from recent infection, new cases from past infection and relapses from previous tuberculosis. In the same year the total number of known active cases was 101,637 and the mortality for tuberculosis was 2.1 per 100,000 inhabitants.

With this information on hand, we may turn our attention to the question of control. The objectives of the tuberculosis control program are: 1) to reduce or eliminate the probability of the non-infected becoming infected; and 2) to prevent the infection from progressing into overt disease in the already infected person.

The ideal method of protecting the non-infected sector of the population is to induce a state of specific immunity through vaccination. Unfortunately a satisfactory vaccine is not available. Although the vaccine BCG has been demonstrated to have some effectiveness (*91*), its value is greatly outweighed by the methods of tuberculosis control. In the U. S. the thrust of the tuberculosis control program is centered on breaking the chain of transmission. This requires vigorous surveillance of existing cases and the finding of new cases so that they can be treated and rendered non-infectious. About 85% of the newly diagnosed cases are in a moderately or far-advanced stage and have infected a certain number of contacts before — not after — a diagnosis (*31*). These newly infected persons (new tuberculin converters) add to the future reservoir of tuberculosis. Because of the high risk of this group's having tuberculosis (3,000 cases in 70,000 new reactors or about 4,300/100,000 new cases), they have first priority for preventive treatment (chemoprophylaxis). Public Health Service trials that were started in 1955 demonstrated that chemoprophylaxis with isoniazid reduces by 50% to 80% the incidence of tuberculosis in high risk groups. Next in order are the tuberculin reactors with abnormal X-rays without previous diagnosis of tuberculosis, and finally the tuberculin reactors with normal X-rays. The latter group added 23,000 new cases of tuberculosis in 1971, which represents 58.2% of all new cases reported in the same year. Systematic chemoprophylaxis is impossible to carry out mainly because of the large number of people (13,900,000) in this category. In the absence of a procedure, laboratorial or otherwise, to determine which of these 13,900,000 are likely to develop tuberculosis, it seems that eradicating tuberculosis will take many years with the current diagnostic techniques. Research to develop procedures for identifying the high risk individual from the total population of tuberculin reactors is thus of utmost importance.

The foregoing discussion clearly indicates that a tuberculosis control or eradication program depends upon the existence and continued up-dating of the kinds of data that are illustrated in the diagram in Figure 9. Collected on a yearly basis, the data may be plotted to give an overview of the trends in the tuberculosis problem. Figures 10 and 11 are examples of graphic representations of tuberculosis in the U. S.

The incidence of tuberculosis in the U. S. is low and is falling; most of the new cases come from the reservoir of an estimated 16,000,000 tuberculin reactors. The new infections (see left side of Fig. 9) that occur every year constitute a group whose detection and treatment is of primary importance. The new case is a source of infection before diagnosis is made and treatment is started, and emphasis on examination and preventive treatment of the infected contacts can prevent future cases. Each new case may start a small epidemic, and the finding of these small epidemics of tuberculosis is an essential part of the control of this disease. The following are illustrative examples adopted from the *Morbidity and Mortality Weekly Report* published by the U.S. Department of Health, Education, and Welfare, CDC, Atlanta, Georgia.

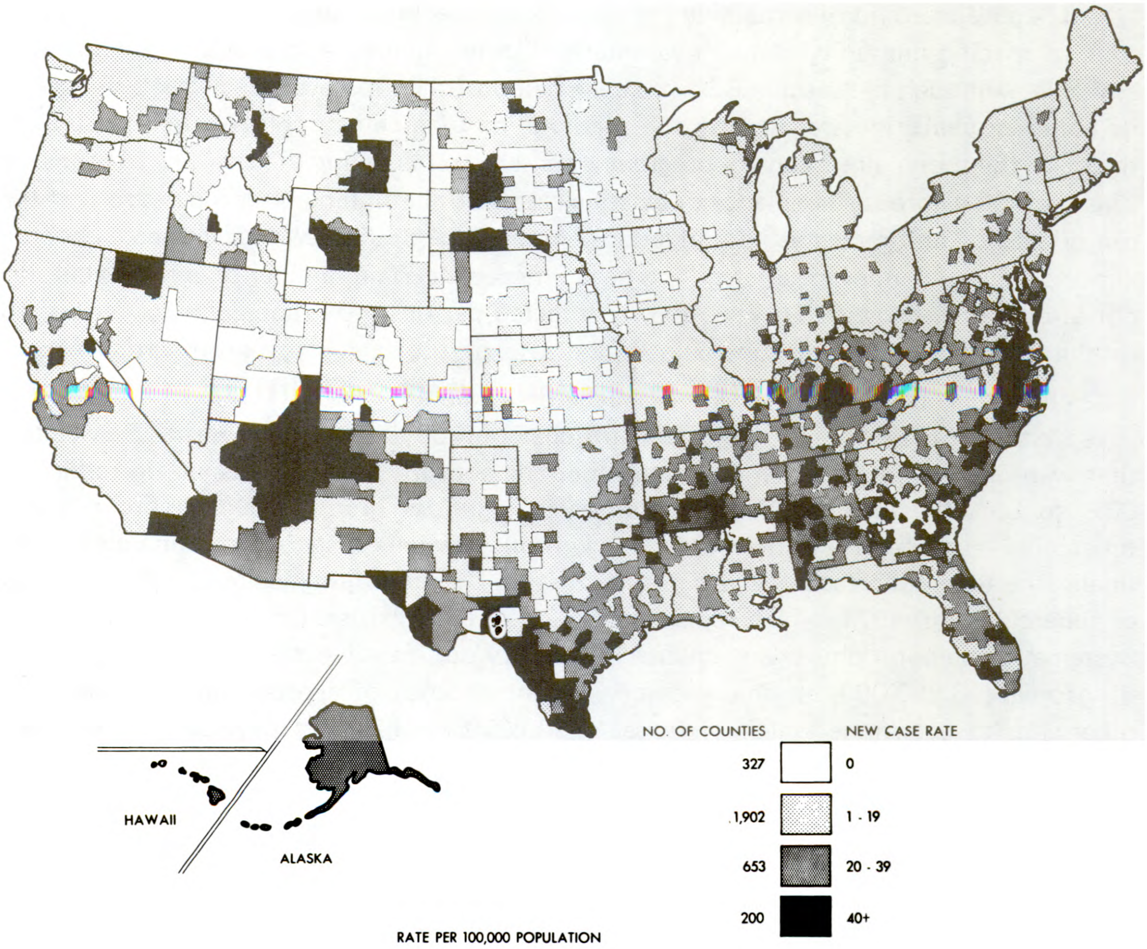

FIGURE 10. New active tuberculosis cases per 100,000, by county, average, 1969-1971 (USPHS, DHEW, CDC).

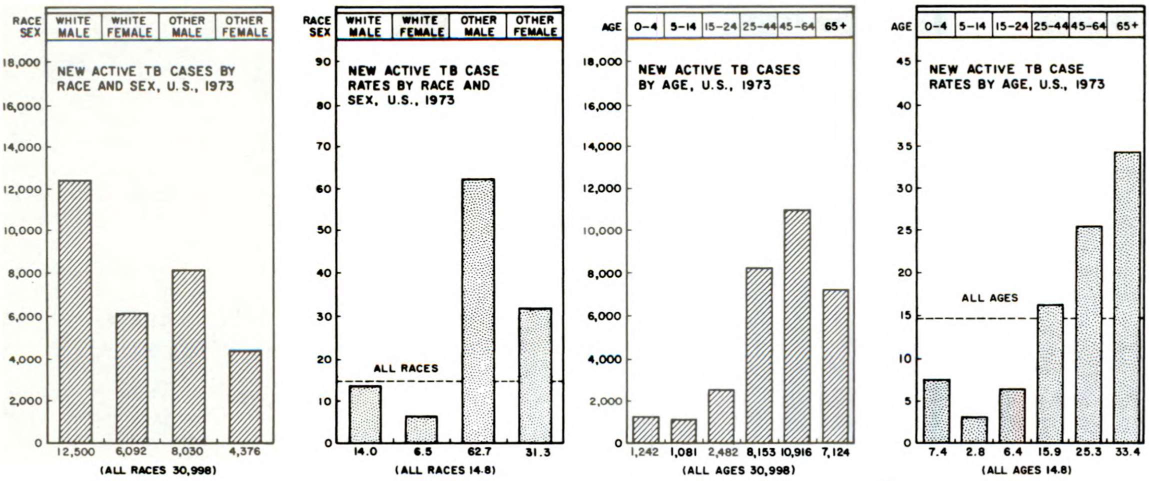

FIGURE 11. New active tuberculosis cases and case rates by age group, by sex and color, United States, 1973 (USPHS, DHEW, CDC).

Example 1
TUBERCULOSIS – Tennessee
(MMWR, Vol. 19, No. 21, 1970)

In January 1969, a 35-year-old animal handler, who worked for a veterinarian in Knoxville, Tennessee, was found to have a positive tuberculin skin test of 13 mm. He had had a negative skin test in September 1968. Chest X-rays in January and again in July 1969 were also negative. The man was started on 300 mg of isoniazid daily for 1 year. In January 1970 after the patient's year of treatment, he was retested and remained positive. All members of his family were also skin tested and were negative.

The patient's only known exposure to tuberculosis had been to a squirrel monkey, treated as an outpatient for pulmonary disease at the veterinary hospital in late August 1968. The monkey coughed frequently and directly on the patient during some of the procedures in the pet hospital. The monkey died after 1 week of treatment, and a necropsy demonstrated lesions compatible with miliary tuberculosis. Culture material yielded *Mycobacterium tuberculosis*.

The monkey had been purchased in August 1968 from a pet shop in Maryville, Tennessee. The monkey had been in the pet shop 2 weeks prior to sale and then in the home of the new owner for 2 weeks before it showed signs of the respiratory illness.

When necropsy suggested tuberculosis, the veterinarian, the animal handler, the receptionist at the veterinary hospital, the monkey's owner, and the owner of the pet shop were tuberculin tested. All were negative. In a retest in January 1969, only the animal handler had become positive.

(Reported by Mary Duffy, M.D., Director, Knox County Health Department; Luther E. Frederickson, D.V.M., Public Health Veterinarian, Tennessee State Department of Health; R. D. Linnabary, D.V.M., Chapman Highway Animal Clinic, Knoxville; and the Microbacteriology Unit, Diagnostic Services, Animal Health Division, U.S. Department of Agriculture, National Animal Disease Laboratory, Ames, Iowa.)

Editorial Comment:

Tuberculosis is well recognized as a public health hazard encountered in Old World monkey species, but its occurrence is rarely reported in New World species frequently kept as pets *(1-3)*. This species has been thought to be refractory to tuberculosis and is not routinely tuberculin tested for this reason.

References

(1) Fiennes, RN: Incidence of T.B. in squirrel and marmosets. *Lab. Primate Newsletter 4*:10, 1965.

(2) Chrisp, LE, Cohen, BJ, Ringler, DH, Abrams, GD: Tuberculosis in a squirrel monkey (Saimiri sciureus). *J Amer Vet Med Ass 153*:918-922, 1968.

(3) Hessler, JR, Moreland, AF: Pulmonary tuberculosis in a squirrel monkey (Saimiri sciureus). *J Amer Vet Med Ass 153*:923-927, 1968.

Example 2
TUBERCULOSIS – Florida
(MMWR, Vol. 20, No. 19, 1971)

In September 1969, a 65-year-old man was admitted to a hospital in Miami with pulmonary edema secondary to hypertension and coronary artery disease. He spent 3 hours in the emergency room, 57 hours in a 45-bed ward, and 67 hours in a smaller, 4-bed special care area where he died. Postmortem examination revealed active pulmonary tuberculosis with massive caseation.

Due to the advanced state of the patient's disease and the possibility of significant spread to exposed hospital personnel, 102 hospital employees, who were tuberculin negative in 1968, were tested in October 1969 with the Mantoux tuberculin test using PPD intramuscularly. Twenty-six of the 102 (25 percent) converted to positive, suggesting recent exposure. (In 1968, 3.2 percent of previously negative hospital personnel in contact with patients had been tuberculin positive.) Two of the 26 employees with positive tests were actively symptomatic. One was a nurse's aide who had radiographic evidence of pulmonary tuberculosis and a positive sputum culture. The other, a house officer, had a negative chest X-ray but had afternoon sweats, fever, and weight loss.

A similar group of 20 hospital employees, tuberculin negative in 1968, with no known exposure to the patient was skin tested, and only one of these had a positive reaction. All 20 were employees who came in close and prolonged contact with other tuberculosis patients.

Further investigation showed that those working on the 45-bed ward with minimal exposure to the patient had a conversion rate of 13 out of 44, whereas similar personnel working in the 4-bed ward showed a rate of 0 out of 12. The 45-bed ward has central air-conditioning that recycles 70 percent of the air, with no bacterial filter. The 4-bed ward, although air-conditioned, has wall units that are 40 feet away from double doors opening to the outside. These doors are opened frequently or left open, thus exposing the room to outside air.

Although unknown differential exposure to tuberculosis patients might account for the differences in conversion rates of these two groups, the available evidence suggests airborne spread of tubercule bacilli throughout the air-conditioning system in the 45-bed ward.

(Reported by Leilani Kicklighter, R.N., Nurse Epidemiologist, Jackson Memorial Hospital, Miami, Florida; N. Joel Ehrenkranz, M.D., Professor and Acting Chairman, Department of Epidemiology and Public Health, University of Miami School of Medicine, Florida; Milton Saslow, M.D., Director, Dade County Department of Public Health; and E. Charlton Prather, M.D., Chief, Bureau of Preventable Diseases, Florida State Division of Health.)

Example 3
TUBERCULOSIS – Nebraska
(MMWR, Vol. 21, No. 31, 1972)

Between July and October 1971, three cases of tuberculosis occurred among relatives in southwest Nebraska. Case 1 was a 53-year-old man living in Lewellen, Nebraska. On July 28, he was admitted to a hospital in Omaha with far advanced pulmonary tuberculosis. He died in the hospital on Sept. 13, 1971. The patient's 8-month-old grandson (Case 2) from North Platte, Nebraska, was hospitalized in Denver, Colorado, on Sept. 16, 1971. He had been ill for approximately 1 month. A diagnosis of tuberculosis meningitis was made. The patient was discharged on Oct. 15, 1971, and has subsequently recovered. Case 3 was the 40-year-old son-in-law of Case 1, also from Lewellen. On Oct. 14, 1971, he was admitted to a hospital in Scottsbluff, Nebraska, with moderately advanced pulmonary tuberculosis and active laryngeal tuberculosis. On October 22, he was released from the hospital on isoniazid and rifampin. It is believed that Cases 2 and 3 were contacts of Case 1, but this could not be documented. However, Cases 1 and 3 lived in Lewellen, and Case 2 received routine medical care there.

Of the 76 close family contacts of these cases, 70 were tuberculin tested, and eight were identified as reactors and started on isoniazid prophylaxis (INH). Two close contacts who were not reactors were also given INH. Tuberculin skin-testing of the entire town of Lewellen and some testing in two nearby towns was conducted. In Lewellen, 411 people were skin-tested, 404 (98.3%) were read, and 18 (4.5%) were identified as reactors. In the other two towns, an additional 528 people were tested, 526 (99.0%) were read, and four (0.8%) were identified as reactors. Of these 22 reactors, 16 (72.7%) were started on INH. The final compilation shows that 1,009 people were tested, 1,000 (99.1%) were read, and 30 (3.0%) were identified as reactors; of these, four adults and two students were documented recent converters. Of the total 30 reactors, 21 (70.0%) were started on INH.

(Reported by F. Lee Szynskie, Public Health Advisor, and Henry D. Smith, M.D., Director of Health, Nebraska State Department of Health.)

Mycobacterium bovis

In many textbooks, *M. bovis* is treated as a variety of *M. tuberculosis* and is referred to as *M. tuberculosis* variety *bovis* (Bergey et al. 1934) or *M. tuberculosis typus bovinus* (Lehmann and Neumann, 1907). These organisms were distinguished by laboratory means by Theobald Smith (*75,76*), and because their isolation from humans has distinct epidemiological and public health significance, they must be treated separately.

The *M. bovis* cells are identical to those of *M. tuberculosis*, either in size, shape or intensity of coloration in the Ziehl-Nielsen's or fluorochrome staining procedures, but they tend to be shorter (0.5 to 1.5 μ) when recently isolated. Cording is not as distinct in *M. bovis* as in *M. tuberculosis*. Growth is visible in 5 to 7 weeks of incubation at 36–37°C and is best in media without glycerol. Glycerol containing media are usually inhibitory on primary isolation (*76a*), and an egg medium with pyruvate replacing glycerol is recommended for primary isolation (Stonebrink medium, *81*). However, the media usually used in the diagnosis of tuberculosis (Lowenstein-Jensen and the Middlebrook and Cohn's 7H10 medium) can be used for isolation of *M. bovis* provided glycerol is omitted. The organisms grow very slowly on these media, yielding small (1.0 mm in diameter), translucid, colorless, pyramid shaped, rough or granular colonies. When compared to the colonies of *M. tuberculosis* on the same media, they are smaller and are said to be *dysgonic* (*M. tuberculosis* colonies being *eugonic*).

M. bovis differs from *M. tuberculosis* in the following properties: niacin test negative; nitrate reductase test negative; usually resistant to PZA; sensitive to TCH. The two organisms have distinctive pathogenicity for laboratory animals.

M. tuberculosis is pathogenic for the guinea pig and seldom for the rabbit, whereas *M. bovis* causes progressive and fatal disease in both. The histopathological aspect of the lesions is indistinguishable. In man, *M. bovis* causes disease which in no way differs from the disease caused by *M. tuberculosis. M. bovis* may be transmitted in man in the same way as *M. tuberculosis;* however, most often infection is acquired by the ingestion of contaminated food, mainly raw milk. Thus, diagnosis of *M. bovis* disease in man must always be followed by an investigation to determine if it can be traced back to the consumption of contaminated milk and to infected herds. The incidence of *M. bovis* mycobacteriosis in man in the U. S. is low (from 1969 to 1971, the CDC laboratory identified only 21 strains), and this is also true in other areas of the world.

The anatomical localization of *M. bovis* tuberculosis (mycobacteriosis *bovis*) in man appears to relate to its usual portal of entry in the alimentary tract. As shown in Table 7, most cases of the *M. tuberculosis* mycobacteriosis are located in the lungs and bronchial lymph nodes (airborne mode of transmission), whereas most cases of the mycobacteriosis *bovis* are localized in the abdominal organs (food borne mode of transmission).

Except for pyrazinamide, *M. bovis* is sensitive to the current anti-tuberculous chemotherapeutic agents, and the disease responds well to therapy.

BCG (Bacille de Calmette et Guerin) is an attenuated mutant derived from *M. bovis*. This attenuated variant was obtained after several years of subculture of M. bovis on a glycerol-bile-potato medium (*7*). It exhibits the same *in vitro* reactions as *M. bovis*. Growth on solid media is eugonic.

The strain BCG is used as a vaccinating agent in most countries of the world, and is recommended by the World Health Organization in their tuberculosis control programs (*91*).

The vaccine is usually given by the intracutaneous route. Tuberculin testing is recommended before BCG is injected; otherwise, Koch's phenomenon may be induced. BCG vaccination elicits a tuberculin type of allergy.

TABLE 7. The Relative Frequency of the Human and the Bovine Type of Bacillus in 2,227 Cases of Human Tuberculosis [adapted from Rich, *65*, page 58]

Site of Lesions	Children Up to 16 Years		All Over 16 Years		Total Adults and Children		Total Cases
	Human Type	Bovine Type	Human Type	Bovine Type	Human Type	Bovine Type	
"Abdominal Organs"	70 (59.9%)	47 (40.1%)	36 (76.6%)	11 (23.4%)	106 (64.7%)	58 (35.3%)	164
Skin (lupus only)	21 (58.4%)	15 (41.6%)	45 (81.9%)	10 (18.1%)	66 (72.6%)	25 (27.4%)	91
Cervical and Axillary Lymph Nodes	105 (66.1%)	54 (33.9%)	65 (97.0%)	2 (3.0%)	170 (75.3%)	56 (24.7%)	226
Bones and Joints	108 (71.1%)	44 (28.9%)	50 (98.0%)	1 (2.0%)	158 (77.9%)	45 (22.1%)	203
Generalized Tuberculosis	244 (89.4%)	29 (10.6%)	25 (96.0%)	1 (4.0%)	269 (90.0%)	30 (10.0%)	299
Meninges	44 (91.7%)	4 (8.3%)	7 (100%)	0 (0.0%)	51 (93.8%)	4 (6.2%)	55
Urogenital Organs	2 (100%)	0 (0.0%)	36 (97.3%)	1 (2.7%)	38 (97.5%)	1 (2.5%)	39
Lungs and Bronchial Lymph Nodes	88 (97.8%)	2 (2.2%)	1,057 (99.7%)	3 (0.3%)	1,145 (99.6%)	5 (0.4%)	1,150
Total	682 (77.8%)	195 (22.2%)	1,301 (97.8%)	29 (2.2%)	2,003 (88.9%)	224 (11.1%)	2,227

Mycobacterium avium-intracellulare

M. avium-intracellulare cells are pleomorphic, but usually are short rods with bi-polar acid fast granules and do not form cords. On solid egg media these organisms form small, circular, dome-shaped colonies with shades varying from nonchromogenic to yellowish pigment as they mature. The Sm D colonies are opaque, yellowish, and about 1.5 mm in diameter, and they are usually composed of very small, 1.2 x 0.5 μ cells; in the smaller and transparent colonies of 0.5 mm in diameter, also Sm D cells may be larger, 1.6 μ in length. Smooth T colonies are thinly spread, transparent, with irregular edges. Rough R colonies may also be seen. They grow slightly faster than *M. tuberculosis,* but the difference in growth rate is not significant enough to be of diagnostic value. The organisms must be distinguished from other non-chromogenic, slow growing mycobacteria such as *M. xenopi, M. triviale, M. gastri, M. terrae* and other still unclassifiable Group III mycobacteria. *M. avium-intracellulare* are niacin negative, nitrate reductase negative, catalase 68°/pH 7.0 positive, Tween 80 hydrolysis negative, reduce tellurite within 3 days, do not grow in 5% NaCl medium and are arylsulfatase negative. Strains that yield a smooth type of growth can be further typed by agglutination reactions according to Schaefer (see page 19).

For practical reasons, we equate human disease caused by Group III mycobacteria with that caused by *M. avium-intracellulare*. However, it seems that some human cases are also caused by other Group III mycobacteria that cannot be classified by current bacteriological methods.

Most often this mycobacteriosis in the U. S. is a chronic cavitary disease of the lung seen in white males over 40 years of age living in rural areas (*14*). It is a disease of slow progression and mild symptomatology. Pneumoconiosis, silicosis in particular, and chronic bronchitis may be predisposing factors. Because these bacteria exhibit multiple drug-resistance, chemotherapy as a rule is unsuccessful. Resection therapy is favored when the extension of the lesions may allow it.

Other forms of the disease described are adenitis, skin lesions, kidney involvement, osteomyelitis and disseminated mycobacteriosis.

A purified tuberculin-like protein (PPD-B) prepared from the Battey Strain CDC No. 100616 was used in skin testing surveys in the U. S. As judged from the results of these surveys, human infections by these bacteria are predominantly seen in the southeastern states. Also, the mycobacteriosis caused by these agents is more often encountered in these same states. One will note that the prevalence of infection is much higher than morbidity.

The mode of transmission is unknown. As a rule, the disease is not transmitted from man to man even with close familial contacts. To date, reports on the isolation of these organisms from the environment (soil and farm animals like hogs and chickens, cattle, and milk) do not furnish any clues to the source of infection. However, a series of reports by Australian workers (*64*) link infection in hogs to infection in humans in a difficult-to-question fashion. On the other hand, European students have reported *M. avium* infections in man more often than they recognize *M. intracellulare*, possibly because of the more frequent virulence of the European isolates to chickens. In the absence of a clear-cut decision on the identity of these bacteria, and the lack of systematic serotyping as proposed by Schaefer, it is difficult to interpret the data in the literature.

Like other Group III mycobacteria, *M. avium-intracellulare* may be isolated from the secretions of normal individuals. Since *M. gastri, M. triviale* and *M. terrae* have been rarely incriminated in human disease, their laboratory identification is important. On the other hand, the isolation of *M. avium-intracellulare, M. xenopi* (discussed later on) and unidentified non-chromogens of Group III requires further study. These are the potentially pathogenic Group III mycobacteria, and the following may be acceptable criteria to link their isolation to actual disease:

1. Absence of mixed culture with *M. tuberculosis* or *M. bovis.*
2. Repeated isolation of the same organism.
3. Isolation from a closed lesion.

Mycobacterium xenopi

Mycobacterium xenopi cells are usually long (4-5 μ to 7-10 μ) and thin, and do not exhibit intense acid fastness. On solid Middlebrook and Cohn 7H10 medium they form very characteristic colonies after 3 to 4 weeks of incubation. These are small and granular, with a peripheral network of filaments (Sm X on Rg X colonies), and some may form aerial hyphae that partially or completely cover mature colonies. The organisms grow at 37°C, but their optimal temperature of growth is around 42°C; they do not grow at 45°C. They are niacin negative. The cultural properties of *M. xenopi* are those of Runyon's Group III mycobacteria, and it is differentiated from the other species on the basis of the following reactions: nitrate reductase negative, Tween 80 hydrolysis negative; 3-day tellurite reduction negative; and a strong 2-week arylsulfatase reaction.

M. xenopi is the causative agent of a chronic progressive pulmonary disease which is clinically and radiologically similar to tuberculosis. It has been found especially in England and Wales, but was reported in France, Holland, Denmark, Belgium, and the U. S. The disease is progressively fatal without treatment, but chemotherapy is often successful. The bacteria are noderately sensitive to INH (growth at 0.2 μg/ml but not at 1.0 μg/ml) and sensitive to STR., ETH., D-CS and KANA; it is often resistant to PZA and ETA.

The mode of transmission is unknown. In England and Wales it is found in towns on the south and west coasts, but the available information does not seem to establish any geographic predominance. The organism recognized as a human pathogen in England and Wales was called *M. littorale* (*48a*) until it was found to be identical to a pathogen of the frog *Xenopus laevis* which Schwabacher described in 1959 (*68*) and called *M. xenopi*. Its normal habitat is unknown and the toad infections, like those in humans, may be opportunistic.

Mycobacterium kansasii

M. kansasii cells are long and thick, and when seen in pathologic specimens are often barred in a characteristic fashion. On solid media they form Sm K and occasionally form rough colonies that are buff-colored if grown in darkness, but turn bright orange-yellow when exposed to visible light (photochromogen). Pigment synthesis is induced in young, actively growing cultures by exposing them to visible light and then incubating them with free access of air. They are niacin negative and are further identified by a positive nitrate reductase test, usual resistance to kanamycin and sensitivity to amithiazone (Tb_1), and negative results with the 2-week arsylsulfatase test. They must be differentiated from *M. marinum*, also a photochromogen.

In man, the mycobacterioses *kansasii* (or *luciflavoses*) is a disease of the lungs clinically and radiologically indistinguishable from tuberculosis. Often the disease is diagnosed in routine radiographic examinations or after an episode of haemoptysis, or it may express itself with the usual symptoms that are suggestive of pulmonary tuberculosis (coughing, general weakness, chest pain, and so forth) that may continue for weeks or months.

Like tuberculosis, the disease may be manifested in other less common localizations. Adenitis, infection of the genitourinary tract, and disseminated disease have been reported. Histologically the lesions are similar to tuberculosis, but are usually more exudative. Some reports of chronic skin granulomas caused by *M. kansasii* were confirmed in the CDC laboratory.

M. kansasii infection is more frequent in urban and suburban areas, particularly in Texas, California, and Chicago. It appears in patients belonging to the higher socio-economic groups, and there is no evidence of person-to-person transmission.

The natural habitat of *M. kansasii* is unknown. The organisms have been isolated from swine and cattle. Most attempts to isolate the organisms from water and milk have failed. However, the successful isolation of *M. kansasii* from water was recently reported.

Mycobacterium marinum

M. marinum cells in the tissues are usually sparse and the bacilli must be sought carefully.

In most instances, subcultures of *M. marinum* grow on solid media within 7 days of incubation. The optimal temperature of growth is 30°C, but they grow at 36° to 37°C, especially on subcultures. The temperature requirements are of particular importance when isolation from skin lesions is attempted. The usual colony types on the Middlebrook and Cohn's 7H10 medium are the Sm D and Sm S; the Smooth Kw colony characteristic of *M. kansasii* is occasionally seen. Rough colonies occur infrequently. The colonies are buff colored if the cultures are incubated in darkness but are orange-yellow if exposed to visible light when young (photochromogenic mycobacterium). *M. marinum* must be differentiated from *M. kansasii*, another photochromogen.

M. marinum is the etiologic agent of a chronic granuloma of the skin in man. Usually there is a history of a draining skin lesion of some weeks or months' duration. With or without reference to a superficial trauma, the lesion may have begun as a small, red, non-tender papule that enlarged slowly, opened, and drained a small amount of pus. The lesion may then progress to an ulcer that is limited to the skin and subcutaneous tissue. A regional adenopathy is not always present. On occasion the disease may simulate lymphatic sporotrichosis (a disease by the fungus *Sporotrichum schenkii*), in which case the lymphatics draining the lesion are cord-like (lymphagytis) and chains of subcutaneous nodules develop. The lesions are most often located in the arms or the legs. The infection responds slowly and poorly to chemotherapy. Surgical excisions seems to be the treatment of choice.

The "mycobacteriosis balnearia" is common in the U. S. and often is associated with exotic fish grown either in fresh water or salt water tanks. Otherwise, epidemics or isolated cases have been associated with swimming pools (from which the designation "swimming pool granuloma" comes) and the coastal areas along the Gulf of Mexico. In some cases, ecological relationships in the occurrence of a trauma are not apparent. As is true for all the other mycobacterioses, the incidence of infection, as judged by skin test surveys with PPD-P, is much higher than the morbidity.

Mycobacterium scrofulaceum

M. scrofulaceum cells are pleomorphic. On solid medium the organisms form visible colonies in 2 to 3 weeks of incubation at 36-37°C. The cell mass is yellow or orange-yellow whether the organism is grown in darkness or is exposed to visible light (scrotochromogen);

the intensity of the color is nonetheless enhanced by continuous light exposure and it may turn red-orange. Crystals of pigment are rarely seen. The colonies are usually smooth, circular, pigmented, and easily emulsified. Sm S or Sm D colonies are more frequently observed on the Middlebrook and Cohn's 7H10 medium. The organisms must be differentiated from other scotochromogens such as *M. gordonae* and *M. flavescens.* The properties useful in the diagnosis of *M. scrofulaceum* are: a negative nitrate reductase test; a Tween 80 hydrolysis negative test; it is 2-week arylsulfatase negative; and it does not grow on 5% sodium chloride ATS medium.

In humans, especially in children, the mycobacteriosis *scrofulaceum* is often a disease of the lymphatic system, most frequently submandibular adenitis. The portal of entry is possibly in the oropharyngeal region. On sections, the nodes are more often purulent in this disease than in tuberculosis, but the lesions are still very similar to those noted in tuberculosis.

In adults, *M. scrofulaceum* may colonize old tuberculosis cavities of the lungs, in which case the repeated isolation of the organism is suggestive of secondary invasion.

The tuberculin-like protein from *M. scrofulaceum* was prepared from a strain named Gause and is designated PPD-G.

The mode of transmission of the infection is unknown, as is the natural habitat of the organism.

Mycobacterium fortuitum

M. fortuitum cells are pleomorphic and exhibit various degrees of acid fastness. As viewed in the pathologic specimen, they may be grouped in palisade formation, and many non-acid fast cells are usually apparent. Unlike other mycobacteria, *M. fortuitum* causes pus formation (coliquative necrosis) rather caseation (coagulative necrosis). These organisms grow on most common bacteriological media, and on Middlebrook and Cohn's 7H10 medium, they form large and usually rough colonies (rough-R colonies), but Sm S colonies are also seen. Growth in most media is apparent in less than 7 days of incubation at 36-37°C (rapid grower, Runyon's Group IV). The colonies are buff or colorless. The organism is differentiated from other rapid growers by the following properties: ability to grow on either MacConkey's agar and 5% sodium chloride ATS medium, and a strong 3-day arylsulfatase test. Most strains reduce nitrates to nitrites, but this property is variable. Among the organisms exhibiting the above properties, some investigators (*62*) feel that two species can be recognized: *M. fortuitum*, which are nitrate reductase positive and capable of growing on 5% sodium chloride ATS medium, and *M. chelonei*, which are nitrate reductase negative and unable to grow on 5% sodium chloride ATS medium. Additional tests reported to be of differential value are the iron uptake test and the ability to utilize fructose.

In this laboratory, those properties may vary even in single strains to such an extent that it is often doubtful that it can be assigned to one or the other species, particularly when recent isolates are under study.

M. fortuitum may be typed by using three bacteriophages. However, the bacteriophage typing of organisms that cause human infections only sporadically is of doubtful eipdemiological significance.

The most common clinical condition is an abscess at the site of a trauma, in many instances the site of injection of supposedly sterile products; less frequent are pulmonary abscesses, corneal ulcers, meningitis, and infections complicating surgery. Since the organism has been isolated from soil, water, and house dusts, the correlation of abscess formation and trauma appears to be a satisfactory explanation in many instances (iatrogenic infections). The etiology and pathogensis of lung disease are obscure, but the disease appears to develop upon predisposing conditions such as achalasia, emphysema, previous tuberculosis, and lipid pneumonitis (*55a*).

Chemotherapy of *M. fortuitum* disease is unsuccessful, because the organisms are resistant to most etiotropic drugs, with the possible exception of erythromycin. Surgery may be indicated; the opening and free drainage of abscesses is successful as a rule.

Mycobacterium ulcerans

M. ulcerans bacilli are 3 μ long by 0.2-0.35 μ wide, although they may be shorter (0.75 μ) or longer (12 μ). Seen in the pathologic specimens they may appear in clumps or singly, and most are extracellular, usually large numbers of bacilli are seen. They grow very slowly, and the cultures must be incubated for at least 3 months; primary isolation of some strains may require 9 months of incubation. The optimal temperature is 30°-33°C; the organisms will grow poorly or not at all at 25° or 37°C and will not grow at 41°C. The colonies are irregular, usually low convex, moist or rough. The colonies are pale cream or yellow.

M. ulcerans causes a chronic, indolent, extensively spreading and crippling ulceration of the skin. The necrotic process may involve the muscles. The ulcers may heal slowly in months or years, or the infection may extend widely and lead to gross deformities. It is most often located on the exterior surface of the limbs but can locate in the abdomen, thorax and face. There is no glandular involvement. In spite of the extensive appearance of the ulcer, the patients are usually in good general health. Treatment is by surgery, which consists of the radical excision of the diseased tissues; plastic surgery may be necessary.

The disease was described first in Victoria, Australia (*63*), and was later found in Malaya, New Guinea, Congo, Nigeria, and Uganda (*15,16,47,83*). The organisms from Buruli (Uganda) were at first thought to be different from *M. ulcerans* and were called *M. buruli.*

Mycobacterium africanum

Strains of mycobacteria isolated from pulmonary tuberculosis in Africa (Nigeria, Congo, Cameroon) that differ in some respects from both *M. tuberculosis* and *M. bovis* have been reported recently. To these organisms the epithet *M. africanum* was proposed, but it has not yet been accepted. These organisms share properties of *M. tuberculosis* and *M. bovis.*

The disease caused by so-called *M. africanum* is indistinguishable from tuberculosis, and appears to respond well to standard chemotherapy. Because many of the strains are resistant to the thiosemicarbazones often used in Africa, this type of drug may not be of value in the treatment of these patients (*13*).

Mycobacterium leprae

Seen in the tissues, *M. leprae* (Hansen, 1874; Lehmann and Neumann, 1896) cells are uniformly stained rods, 1 to 8 μ long and 0.2 to 0.5 μ wide. The rods may be straight or slightly curved, but variations in size, shape, or uniformity of staining are frequently seen. The cells may be beaded, and may appear as acid-fast granules. In active cases the bacilli are arranged in "cigar shaped" bundles, and in some advanced cases, clumps of bacilli are within disk-like masses or globs. The organisms are less acid fast than the tubercle bacilli. *M. leprae* has not been successfully grown in laboratory media, but when inoculated into the mice foot pads, the organisms divide slowly (*69*); the average generation time is about 12½ days (*71*). The successful transfer in the mice foot-pad made it possible to study the effect of chemotherapeutic agents in a laboratory model (*70,72*).

The bacteriological diagnosis of leprosy consists of demonstrating the bacilli in smears from the skin lesions or in scrapings from the nasal mucosa. The skin area to be examined is thoroughly cleaned and pinched up. With a scalpel an incision well into the infiltrate is made, and some tissue from under the epidermis is obtained by scraping. Scrapings of the nasal mucosa are taken on the internal septum with the aid of a scalpel.

Leprosy is a disease of insidious onset. The incubation period may be a few months to several years, usually 2 to 4 years. The bacilli supposedly enter the skin, and at first no tissue reaction develops. According to one hypothesis (*17*), as the disease progresses a cellular reaction gradually develops from an undetermined stage (borderline leprosy) to a florid, well established granulomatous stage (tuberculoid leprosy). There are intermediate stages that correspond to a gradual build up of immunity (lepromatous leprosy).

Leprosy is almost always confined to the skin, mucosal membranes and the nerves, and its clinical expression serves as the basis for various classifications proposed. The marked disability and deformities seen in leprosy are associated with the invasion of the nerve trunks, which constitutes one of the more important elements of the disease. The typical lesion is called a *leproma*, which is a granuloma characterized by a richly vascularized granulation tissue mainly composed of mononuclear cells filled with large numbers of bacilli; in the course of time the cytoplasm becomes vacuolated and acidophilic, and the nuclei are pale (Virchow cells). The tuberculoid granulomas are similar to those in tuberculosis except that the blood capillaries in leprosy remain intact, and the lesions contain very few or no bacilli.

A heat killed suspension of lepromas, rich in bacilli, or lepromin is used as an antigen in skin testing (Mitsuda reaction). The intradermal injection of the antigen elicits a 48 hours reaction (Fernandez reaction) and a late (3 week) nodular reaction (Mitsuda reaction). Lepromatous leprosy is associated with low resistance (negative Mitsuda reaction), while tuberculoid leprosy is associated with more effective immunity (positive Mitsuda reaction).

The disease is worldwide. The total number of leprosy patients in the world has been estimated to be some 35,000,000; the estimated number in the U. S. is 2,000 cases.

Other Possible Pathogenic Mycobacteria

M. simiae (*M. habana, M. asiaticum*) and *M. szulgai* are two newly proposed species alledgely pathogenic to man.

The first species was reported to be an important cause of a tuberculosis-like lung disease in Cuba. It exhibits biochemical patterns similar to *M. avium*, being pigmented (*32a*). The organism can be distinguished serologically from *M. avium-intracellulare.*

M. szulgai was recently described as a human pathogen (*48*) in England; and 4 cases were reported in the U. S. (*67a*). This is a schotochromogen mycobacterium which exhibits the following properties: late Tween 80 hydrolysis positive; nitrate reductase positive; high catalase activity (*68a*).

Experience with either organism is still too fragmentary to warrant further comment.

CHAPTER 2
Laboratory Diagnostic Procedures

Bacteriological Diagnosis of the Mycobacterioses

INDICATIONS AND LEVELS OF PERFORMANCE

The specific diagnosis of the mycobacteriosis requires the isolation of the causative agents in pure culture, and their identification by methods described in Part 1 of this book. Of course, this requirement does not apply to leprosy whose etiologic agent (*M. leprae*) cannot be cultivated in laboratory media. The bacteriological diagnosis unfolds in three successive steps of increasing complexity: 1) demonstration of acid-fast bacilli in the pathological secretions of tissues; 2) isolation of the organisms in pure form; and 3) identification of the isolated organism.

A well equipped laboratory, usually a central (level II) or reference laboratory (level III), is needed to perform all the required procedures. It is impractical and uneconomical, even useless, to have an over-abundance of fully equipped laboratories. Therefore, services needed must be determined and the laboratory equipped to perform the necessary procedures to meet these needs.

When a network of laboratories is organized within a Tuberculosis Control Program, each is assigned one or more of those steps on the basis of laboratory performance, available equipment, and personnel. Laboratories engaged in microscopy alone (peripheral or level I laboratories) are, of necessity, the more numerous; they are invaluable in case-finding. These laboratories must share with the clinicians that use them the responsibility of directing the specimens to laboratories which perform: 1) culture and identification of *M. tuberculosis*, and 2) drug-sensitivity testing for isoniazid, streptomycin, p-amino-salycilic and ethambutol (central or level II laboratories). In turn, these laboratories have the responsibility of referring all mycobacteria that do not conform to *M. tuberculosis* to a reference laboratory (level III). In addition to the responsibility of performing the necessary tests for the final identification of the mycobacteria and performing drug sensitivity tests for all the current chemotherapeutic agents and other related studies, the reference laboratory must be involved in training personnel, evaluating media and reagents, proficiency testing, consultation, and research and development of new diagnostic procedures.

In the U. S. each state has at least one reference laboratory which is technically and administratively part of the State Health Department. These specialized laboratories might be relieved of the handling of clinical specimens as much as practical, relegating this minor aspect of their daily responsibilities. The state laboratory director can request the services of still another reference laboratory which is technically and administratively part of the Federal Government. This laboratory is located in Atlanta, Georgia, at the Center for Disease Control (CDC), a part of the Department of Health, Education, and Welfare. State or county laboratories, private hospitals or laboratories, and individual physicians are not allowed to send specimens directly to CDC unless they are given special permission by the director of their state health department. However, other federal agencies can directly request the services of this reference center.

COLLECTING AND HANDLING CLINICAL SPECIMENS

•Sputum

Instruct the patient on the need to expectorate deep from the lungs. Saliva and nasal secretions are not satisfactory.

Tell the patient to rinse his mouth with water, not mouth washes or antiseptics.

Use clean, sterile, glass or plastic containers with tight fitting closures. Make sure the container is correctly labeled with the name of the patient and the hospital and any other appropriate information.

Collect the specimen early in the morning. Discourage pooled specimens.

A volume that is to 5.0 to 10.0 ml is adequate. However, smaller volumes should also be processed.

Send the specimen to the laboratory. Specimens must be processed within at least 5 days of collection. Therefore, make certain they are mailed as soon as possible. If delays are unavoidable, keep the specimen refrigerated.

•Induced Sputum

Label the container with the patient's name and appropriate numbering system.

Indicate on the label that the specimen is "induced sputum." Since this specimen looks like saliva, laboratories may discard it.

Send the specimen to the laboratory as soon as possible.

•Urine

Collect single, mid-stream early morning specimens. Discourage pooled 24-hour specimens (*34*). Or, collect urine by catheterization of the ureters and label the container accordingly.

Remember that urine is a good culture medium, and even if it is collected aseptically, the specimen is easily contaminated. Therefore, send the specimen to the laboratory as soon as possible; in the meantime, keep it refrigerated.

•Tissues

Collect the tissue aseptically, and place it in appropriate container. *Do not add* a fixative, formalin, or other similar agents.

Send the specimen to the laboratory at once. If a delay is necessary, freeze the tissue and mail it frozen.

•Pus

Pus from abcesses, adenitis, empyema, etc., must be sent to the laboratory at once.

•Other Types of Specimens

Cerebrospinal fluid and fluids in the inflamed serous cavities of the body (pleura, peritoneum, pericardium, and the synovial cavities) must be sent to the laboratory immediately. It is good practice to advise the laboratory of the presence of such specimens. The same applies to menstrual blood or uterine mucosa collected by curettage.

Attempts to isolate mycobacteria from the blood, gastric lavage specimens or feces are of very limited value and should be discouraged.

LABORATORY PROCEDURES FOR MICROSCOPY

•Preparation of Smears

To make *direct smears,* look for chunks of caseous material and thick masses of sputum in the specimen because these are the most likely sources of the mycobacteria. For *concentrated smears,* use a portion of the sediment (see below) to make the smear.

Spread the selected material in a thin layer over an area on the slide.

Always use new slides. Slides previously used may still have attached acid-fast bacteria.

A wire loop can be used for making the smear. It must be dipped into 95% alcohol and flamed thoroughly between specimens.

Small, match-sized sticks may also be used. The sticks should not be used more than once.

The sediment of a concentrated homogenized specimen can be placed on the slide using a pipette or wire loop.

Heat fix the smear.

The smear may be gently dried over a flame, then heat fixed by passing the slide three times through hotter cone of the flame.

An alternative procedure is to heat the smears on a slide warmer for 2 hours at 65° to 70°C (overnight is acceptable).

•Staining Procedures

Ziehl-Neelsen's procedure

□ *Reagents*

Ziehl-Neelsen carbol fuchsin. Dissolve a 0.3 g of basic fuchsin (magenta) in a 10.0 ml of 90%-95% ethanol; add 90.0 ml of a 5% aqueous solution of phenol.

Acid-alcohol. Slowly add 3.0 ml of concentrated hydrochloric acid to 97.0 ml of 90%-95% ethanol, in this order (considerable heat may develop).

Methylene blue counterstain. Dissolve 0.3 g of methylene blue chloride in 100.0 ml of distilled water.

□ *Procedure*

Cover the dried, heat-fixed smear with a small rectangle (2.0 x 3.0 cm) of filter paper. (This holds the stain on the slide.)

Apply approximately five drops of carbol fuchsin to cover the filter paper.

Heat to steaming for 5 minutes. *Do not boil or allow to dry.* If the slide dries, add more stain. The steaming may be done over a Bunsen flame or on a preheated electric staining rack.

Remove the paper with forceps, rinse the slide with water, and drain.

Destain (decolorize) with acid-alcohol until no more stain appears in the washings. (2 minutes)

Counter stain with methylene blue. (1-2 minutes)

Rinse, drain, and air dry. *Do not blot.*

Modified Kinyoun's "cold" procedure

□ *Reagents*

Kinyoun's carbol fuchsin. Dissolve 4.0 g of basic fuchsin (magenta) in 20.0 ml of 90%-95% ethanol, and then add 100.0 ml of a 9% aqueous solution of phenol (9.0 g of phenol dissolved in 100.0 ml of distilled water).

Acid-alcohol and methylene blue. Prepare as for the Ziehl-Neelsen's procedure above.

□ *Procedure*

Proceed as indicated in the Ziehl-Neelsen's technique except allow Kinyoun's carbol fuchsin to stain for 5 minutes *without steaming.*

Auramine fluorescence acid-fast stain

□ *Reagents*

Phenolic auramine. Dissolve 0.1 g of auramine 0 in 10.0 ml of 90%-95% ethanol and then add to a solution of 3.0 g of phenol in 87.0 ml of distilled water. *Store the stain in a brown bottle.*

Acid alcohol. Add 0.5 ml concentrated hydrochloric acid to 100 ml of 70% ethanol.

Potassium permanganate. Dissolve 0.5 g potassium permanganate in 100 ml distilled water.

□ *Procedure*

Cover the dried, heat-fixed smear with carbol auramine and allow to stain for 15 minutes. *Do not use filter paper or heat.*

Rinse with water and drain.

Decolorize with acid-alcohol (2 minutes).

Rinse with water and drain.

"Counterstain" with potassium permanganate (2 minutes).

Rinse, drain, and air dry. *Do not blot.*
The potassium permanganate is not a true counterstain, but does aid in quenching the background fluorescence.

Auramine O-acridine orange fluorescence acid fast stain

□ *Reagents*

Phenolic auramine. Prepare as above.

Acid alcohol. Prepare as above.

Acridine orange counterstain. 0.01 g of acridine orange in 100.0 ml of a 0.1% aqueous solution of anhydrous dibasic sodium phosphate (Na_2HPO_4).

□ *Procedure*

Proceed as for the Auramine O acid-fast staining procedure, except substitute acridine orange (2 minutes) for potassium permanganate. Acridine orange is a true counterstain and gives a brick-red to orange fluorescence to the background (*77*).

□ *Examination of the smears*

Examine smears stained with carbol fuchsin by transmitted light microscopy with oil immersion lens (x100). The bacilli are stained red and the background is blue.

Examine smears stained for fluorescenct microscopy with fluorescent microscopy equipment. The bacilli fluoresce yellow-orange on a dark background (Auramine fluorescent procedure) or on a red to orange background (Auramine O-acridine orange procedure). Make sure the slides are of nonfluorescing glass.

•Classification of Smears

The National Tuberculosis Association classification (*1*) is recommended, and is as follows:

Number of bacilli	*Report*
0	No acid-fast bacilli found.
1-2 in entire smear	Report number found and request repeat specimen.
3-9 in entire smear	Rare or +
10 or more in entire smear	Few or ++
1 or more per field	Numerous or +++

The Gaffky scale is cumbersome and of limited use, but some may find it useful. It is as follows:

Number of bacilli	*Scale*
1 or 4 in whole smear	I
1 on the average in many fields	II
1 on the average in each field	III
2 to 3 on the average in each field	IV
4 to 6 on the average in each field	V
7 to 12 on the average in each field	VI
13 to 25 on the average in each field	VII
About 50 on the average in each field	VIII
About 100 on the average in each field	IX
Innumerable per field	X

In leprosy work, the following scale was proposed (*17*).

Number of bacilli	*Scale*
Many clumps of bacilli in 1 average 1/12 in. objective field (estimated over 1,000 bacilli).	6+
Many bacilli in 1 average 1/12 in. objective field (estimated over 100 bacilli).	5+
10 or more bacilli in 1 average 1/12 in. objective field.	4+
1 or more bacilli in 1 average 1/12 in. objective field.	3+
1 or more bacilli in 10 average 1/12 in. objective fields.	2+
1 or more bacilli in 100 average 1/12 in. objective field.	1+

Note that in this scale each number represents 10 times as many bacilli as the one below it.

•Some Comments on Microscopy

Staining solutions should be stored in brown bottles in a cool location. Light and heat may reduce the staining qualities of a stain or dye.

Many staining solutions will show precipitate after they have been stored. Any stain that shows precipitation should be filtered through glass wool. It may be useful to spot each new batch of staining solution on filter paper, which is then allowed to dry and is stored in a dark container. This spot can be periodically compared with spots of the same solution. The stain must be discarded when there is evident differences in the spots. Periodic disposal of staining solutions may be unnecessary and wasteful; however, it does insure the quality of the stains.

When solutions of the dyes are prepared, correction must be made on content of actual dye. Most reputable manufacturers will stamp each container with the percent of dye. To determine the corrected weight of dye to use, divide 1 by the percent of dye and multiply the quotient by the desired amount of dye. For example:

Need 5.0 g of dye
Dye content = 75%

$$\frac{1}{0.75} = 1.33$$

1.33 x 5 g = 6.65 g are needed

To avoid the transfer of bacilli from one slide to another, never blot dry smears nor use staining jars that hold several slides.

TABLE 1. Proposed Scale for Reporting AFM Results in Comparison with the NTA Scale

NTA Classification				Proposed Scale			
No. of Bacilli Observed	**Report**	**(P)[1]**	**No. of Bacilli[4] Observed**	**Report**	**(P)[1]**	**Expected Concentration of AFB in Specimen**	**(F)[3]**
0	No AFB found	<10%	0/100 or more fields	No AFB found	Less than 10%	Less than 1,000	100
1-2/smear	Report no. found and repeat: (±) or doubtful[2]	44%	1 or more/100 fields	Report no. found and repeat: 1+	50%	About 5,000 to 10,000	100
3-9/smear	Rare or 1+	74%					
10 or more/smear	Few or 2+	97%	1-9/10 fields	2+	90%	About 50,000	50
1 or more/field	Numerous or 3†	98%	1-9/field	3+	96.2%	About 100,000	25
			10 or more/field	4+	99.95% or better	About 500,000	10

[1] Probability of a positive report.

[2] This score is not in the NTA Scale, but was included to account for those reports which the microscopist indicated were in doubt.

[3] Recommended minimum number of fields to screen to classify a smear with the indicated score.

[4] In our experience, the average time spent to study 300 fields is 25 minutes, or 5 seconds per field (about 9 minutes/100 fields; in this study the average time spent in reading smears was 7.4 minutes). We must consider the area screened. The microscopic field is a circle covering an area of about 0.02 mm^2 (0.17 mm in diameter). If the area of the smear is 200 mm^2 (1.0 x 2.0 cm) one must examine 10,000 fields to completely screen the smear. If we conceive of the smear as being divided into squares each with an area corresponding to 100 fields, the total number of squares is then 100. According to the Poisson series, the probability of there being at least one bacillus on a single square is P = 98.8% when 450 bacilli are deposited onto the slide (x = 4.5), or about 138,150 bacilli per ml of specimen. In practice, if only about 1% of the whole smear is screened (100 fields, and if sampling and observation errors are taken into account, the actual number of bacilli must be deposited onto the slide to have the same probability of P = 9.8.% is about 800, which corresponds to about 270,000 bacilli per ml of specimen.

•Sensitivity and Reliability of Microscopy

The following table, adopted from Cruickshank (*19,19a*), gives the levels of sensitivity of microscopy measured against culture.

Ziehl-Neelsen film positive	*Bacilli per ml.*
++++	1,000,000,000 (10^9)
+++	100,000,000 (10^8)
++	10,000,000 (10^7)
+	1,000,000 (10^6)
(+)	100,000 (10^5)
Culture positive	
100 - 1,000 colonies	10,000 (10^4)
10 - 100 colonies	1,000 (10^3)
1 - 10 colonies	100 (10^2)
0 - 1 colony	10 (10^1)
Culture negative	1 (10^0)

In theory, there is no given number of bacilli that cannot be detected by microscopy. What is actually measured is either the probability of finding at least one bacillus, or the probability of two or more microscopists finding bacilli in a specimen containing any given number. The probabilities indicated in Table 1 were empirically determined in our laboratory.

LABORATORY PROCEDURES FOR ISOLATION

•Homogenization and Decontamination of Sputum

NALC-NaOH procedure (*38,39*)

□ *Materials*

50.0 ml sterile centrifuge tubes with water-tight screwcaps.

Sterile pipettes: capillary, 1.0 ml, 10.0 ml.

Microscope slides.

Centrifuge equipped with aerosol-free cups.

□ *Media*

2 to 4 Lowenstein-Jensen (L-J) slants per specimen.

2 7H10 agar medium plates per specimen.

(If only one kind of medium is used, the L-J is recommended.)

□ *Reagents*

NALC-NaOH digestant-decontaminant solution. To make 100.0 ml of solution, combine:

50.0 ml of sterile 4% sodium hydroxide (1.0N)

50.0 ml of sterile 2.9% sodium citrate (0.1M)

0.5 g of a N. acetyl-L-cystein powder

Use this solution within 24 hours. If not used on the day of preparation, store in tightly stoppered container and refrigerate for use the following day. The first and second solutions are sterilized by autoclaving and then stored at room temperature; if a precipitate forms, discard.

Sterile distilled water or phosphate buffer, pH 6.8.

0.2% sterile solution of bovine albumin, pH 6.8 (optimal, see below).

Sterile distilled water in screwcap tubes, 4.5 ml in each.

☐ *Procedure*

1. Transfer the specimen to a centrifuge tube. The volume of the specimen must never be more than one-fifth the volume of the centrifuge tube (10.0 ml of specimen for a 50.0 ml tube).
2. Add an equal volume of the NALC-NaOH digestant.
3. Tighten the cap on the centrifuge tube and mix on a Vortex until liquified (usually 5 to 20 seconds).
4. Let stand at room temperature for 15 minutes.
5. Fill the tube to within 1/2 inch of the top with sterile distilled water, or pH 6.8 phosphate buffer. Tighten cap and swirl the tube by hand to mix.
6. Centrifuge at 3,000 rpm (1,800 to 2,400 x G) for 15 minutes.
7. Pour off the supernatant into a splash-proof can containing a disinfectant.
8. Add 1.0 to 2.0 ml of 0.2% sterile bovine albumin, or sterile distilled water, to the sediment. Shake gently to mix. This is the undiluted sediment, S_1.
9. Take 0.5 ml of S_1 above and add to 4.5 ml of distilled water. This is a 1/10 dilution, S_2.
10. Inoculate two drops of S_1 and of S_2 onto the surface of the L-J tubes (two for each dilution) and onto the surface of the 7H10 agar medium (one plate for each dilution). The inoculum on the surface of the agar medium may be spread with bent glass rods.
11. From S_1 above prepare a smear.

NOTE: When more than one specimen is processed, organize the work in such a way that no specimen is exposed to the digestion-decontamination solution for more than 15-20 minutes (Step 4 above), and start Step 5 as soon as this time limit is reached.

Sodium hydroxide method (Petroff method, *60*)

□ *Reagents*

4% solution of sodium hydroxide

2.0 N HC1: dilute 33.0 ml of concentrated HC1 to 200.0 ml with water (add acid slowly to water and then add water to make the desired 200.0 ml final volume; not the other way around).

Phenol red or bromthyolblue solution

□ *Procedure*

Add to the sputum in a centrifuge tube an equal volume of 4% sodium hydroxyde.

Shake the mixture at intervals; in the meantime keep the specimen in the incubator at 37°C for 15 to 20 minutes.

Centrifuge at 3,000 rpm (1,800 to 2,400 x g).

Decant the supernatant into a splash-proof can containing a disinfectant.

Neutralize the sediment with 2.0 N HC1; add to the sediment one drop of the indicator solution and then the acid, drop by drop, until the color changes to yellow. Avoid excess acid.

Mix the sediment, inoculate the media, and then make a smear.

Trisodium phosphate-Zephiran (*37*)

□ *Reagents*

Digestant solution — add 7.4 ml of 17% Zephiran to 4.0 1 of hot distilled water containing 1.0 kg of trisodium phosphate ($Na_3PO_4 \bullet 12\ H_2O$).

M/15 sterile phosphate buffer, pH 6.6.

□ *Procedure*

Mix equal volumes of digestant and sputum. Shake for 20 minutes, and allow to stand another 30 minutes.

Centrifuge at 3,000 rpm (1,800 x 2,400 g) for 20 minutes.

Discard the supernatant.

Suspend the sediment in 10.0 to 20.0 ml of sterile phosphate buffer.

Centrifuge, and discard the supernatant.

Inoculate the sediment onto appropriate mediums.

Oxalic acid (*18*)

(Useful to treat sputum from patients whose secretions are consistently contaminated with *Pseudomonas* sp.).

Mix thoroughly equal amounts of 5% oxalic acid and sputum in a centrifuge tube; stopper the tube and incubate at 37°C for 30 minutes, shaking occasionally during this time.

Add 10 ml of sterile physiological saline and mix.

Centrifuge at 3,000 rpm.

Decant the supernatant fluid.

Neutralize the sediment.

Inoculate onto culture medium.

Clorox (sodium hypochlorite, *59***)**

(For microscopy only. The tubercle bacilli are killed within 15 minutes after exposure.)

Add equal volume of clorox (active ingredient, sodium hypochlorite) to sputum.

Shake well in mechanical shaker ten minutes.

Centrifuge at 3,000 rpm for 15 minutes.

Decant supernatant fluid.

Smear sediment on slide and allow to dry.

•Other Body Fluids

The spinal fluid and fluids that accumulate in inflamed serous cavities (pleural, pericardial, peritoneal and synovial fluids) are aseptically collected in tightly stoppered sterile test tubes. If the fluid is clear, centrifuge, decant and inoculate the sediment onto the medium. If the fluid is cloudy, mucopurulent, or thick, treat the sediment as for sputum.

•Tissues

Tissues from biopsies, surgical resections, or autopsies are ground in a sterile tissue grinder in sterile 0.85% sodium chloride solution or 0.2% bovine albumin solution, until an even suspension is obtained. Transfer the suspension to a centrifuge tube and treat as for sputum, unless the suspension is known to be sterile, in which case, the centrifugate may be directly inoculated onto the culture medium.

INCUBATION OF CULTURES

•Temperature

Incubate all cultures at 35°-37°C. Cultures from skin lesions must also be incubated at 30°-33°C (*M. marinum* and *M. ulcerans* may not yield growth at 37°C on primary isolation).

- **Atmosphere**

Incubate in an atmosphere of 5% to 10% CO_2 in air.

Slant medium in screwcap tubes: leave the caps loose during first week of incubation, when the caps may be tightened.

Agar base medium in plates: place the plates in CO_2-permeable plastic bags, preferably individually. Seal the bags with tape or a heat-sealing apparatus. Place the plates in the incubator, medium-side down for 2 to 3 days, then invert the plates.

The medium in plates tends to dehydrate and water of condensation accumulates on the lid. This is avoided by placing the plates in individual plastic bags and then placing the plates in cans holding 10 to 12 plates. The cans are then placed in the incubator in which the metallic shelves are covered with an insulating material such as styrofoam or several thicknesses of cardboard.

- **Time of incubation**

Most species pathogenic for humans will grow in suitable media after 3 to 4 weeks of incubation. *M. ulcerans* may require 3 months or more of incubation at 30°-33°C. When tuberculosis is suspected, a culture must not be reported negative before at least 6 weeks of incubation, but more than 8 weeks is not normally required in practice.

ANIMAL INOCULATIONS

For diagnosis, the guinea pig is the animal of choice. The sediment (1-2 ml) of a centrifuged specimen, neutralized if a digestion-decontamination procedure was used beforehand, is inoculated subcutaneously in the right groin. Any suitable size syringe can be used. The site of inoculation must be disinfected and wet; a cotton swab or pledget cotton soaked in 2% phenol may serve the purpose.

Diagnosis is based on the autopsy findings: distribution and appearance of the lesions in the lymphatic nodes, liver, spleen, and lung and the demonstration of acid-fast bacilli in smears from the diseased organs.

Nowadays, guinea pig inoculation has limited indications, and must be referred to specialized laboratories. These indications are:

1. Body fluids in which there are few bacilli (spinal fluid, pleural fluid, etc.).
2. Sputum from patients whose cultures are consistently contaminated.
3. Urine sediment, when cultures are repeatedly negative but the clinical or radiological evidence of tuberculosis of the urogenital tract is present.

Rabbit inoculation does not have a place in diagnosis, and its use in the differential identification of *M. tuberculosis* and *M. bovis* is required only in the very few instances when a strain does not exhibit the characteristic properties described elsewhere in this book. In our laboratories, a saline suspension containing 0.01 mg of bacilli is inoculated intravenously into the marginal vein of the ear. Usually, animals inoculated with this many *M. bovis* cells die after 8 to 10 weeks, but *M. tuberculosis* at this dose never kills the rabbit (*81*).

Drug-Susceptibility Testing

INDICATIONS

The major indications of drug-susceptibility testing are:

Initial cases of pulmonary tuberculosis.

All retreatment cases or relapses.

Primary drug resistance surveillance programs or surveys.

For scientific purposes, therapeutic trials, epidemiological studies, unusual cases, investigations on the nature of drug resistance, studies on the mechanisms of drug action, etc.

The following are *not* indications of drug-susceptibility testing:

Cultures during the initial stages of treatment. The results of the test while the patient is responding to therapy may be misleading. (See pages 129-132.)

Occasional isolate from a quiescent or inactive case.

Cultures of mycobacteria known to be nonpathogenic for man.

LABORATORY PREREQUISITES

For a satisfactory level of performance to be maintained, the volume of work must be reasonably large (more than 10 tests per week). Two important procedures should be followed to assure proficiency in testing:

Perform surface plate counts and determine whether results conform with the Poison law (See Part I, Chapter 4), when preparing cell suspensions.

Run control tests with a standard strain, like the $H_{37}Rv$ strain of *M. tuberculosis,* every time tests are done (bacterial suspensions of known titer in distilled water may be dispensed in Wheaton vials and frozen at -70°C for later use; 1.0 ml of suspension in 5.0 ml Wheaton vials is a convenient volume).

PROCEDURES

•Direct Method

Stain and read smears of concentrated specimens.

Dilute the concentrated specimen on the basis of the smear as follows:

Microscopy	*Dilutions*
Less than 1 AFB/Field	Undiluted and 10^{-2} dilution
1 to 10 AFB/Field	10^{-1} and 10^{-3} dilutions
More than 10 AFB/Field	10^{-2} and 10^{-4} dilutions

Inoculate drug media dispensed in quadrant (Felsen) plates with each of the two dilutions selected. Use three drops from a Pasteur pipette.

Incubate as usual.

•Indirect method

From the surface of the solid medium, transfer a representative sample of the growth, ideally a portion of each colony, to a sterile test tube containing 6-8 glass or plastic beads and 3.0 ml of Tween-albumin or 7H9 broth.

Disperse the cells on a test tube mixer (5 to 10 minutes).

Allow the larger particles to settle. Withdraw the supernatant and adjust the cell suspension to approximately MacFarland No. 1 standard with sterile distilled water or 0.85% sodium chloride solution. (The MacFarland No. 1 standard is prepared by adding 0.1 ml of 1% $BaCl_2$ to 9.9 ml of 1% H_2SO_4).

Dilute to 10^{-2} and 10^{-4} and use both dilutions to inoculate the drug media and controls.

Alternatively:

Use 7 to 10-day-old cultures in Middlebrook and Cohn's 7H9 liquid medium.

Dilute to 10^{-3}, 10^{-4}, and 10^{-6} in sterile distilled water.

Inoculate the drug media and controls with each dilution.

•Recommended Medium

Middlebrook and Cohn 7H10 medium.

•Incubation

Incubate as usual. *Read plates at 3 weeks,* both with the naked eye and with the aid of a dissecting microscope using 30 - 60 x magnification and transmitted light.

•Reporting

From the number of colonies in the control and drug medium, calculate the proportion of survivors. Report this proportion as the percentage of drug resistant cells.

•Interpretation

The report must not be interpretive, and must indicate the actual data.

•Inoculum Size Relationships

1 x 10^8 bacilli weigh approximately 1.0 mg (wet weight). The McFarland #1 = app. 0.1 mg = app. 1.0 x 10^7 bacilli = app. an absorbancy of 0.01 at 650 mm in 7H9 medium.

When an absolute concentration method is used, the inoculum size must not exceed 100,000 bacilli.

•Some Comments on Drug-Sensitivity Testing

The size of the inoculum

The bacterial suspensions of drug-susceptible populations may or may not contain drug-resistant mutants. As noted, these mutants originated from mutations that occur in an unpredictable fashion in growing populations of the tubercle bacilli and were not necessarily present in the pathological specimen from which the original strain was isolated.

As noted before, tuberculosis is essentially an airborne infection. Investigations by Wells and his collaborators (*88,89*) demonstrated that the droplet nuclei were the main factor in the transmission of the disease. More recently Riley and his collaborators (*66*) presented evidence indicating that the number of droplet nuclei initiating a lung infection approaches one, and the probability of infection by one, two, three or more droplet nuclei appeared to follow the already familiar Poisson distribution. It has also been shown that a droplet nucleus transports one to very few tubercle bacilli. Therefore, the tuberculosis infection is initiated by one to very few tubercle bacilli (not by a strain as is often seen in the literature). The consequence of these investigations is that if the infection progresses into disease, the bacterial population in the tissues may be considered as a clone. In a recent cavity, this clone may have the size of 10^8 - 10^9 cells (*11*). During the growth of this clone, mutations may have occurred; some mutants may not have survived, and in some reversions may have occurred. These factors tend to maintain the proportion of mutants at a very low level. A pathological specimen contains a sample of the above population, a portion of which the bacteriologist plates onto a solid medium. A population (a strain) is obtained during the growth of which mutations and reversions may also have occurred. Again, the number of mutants relative to the total population remains low. Bacterial geneticists have investigated the interplay of mutations, reversions and selection in bacterial populations. The interested reader may consult the references for Part 1, Chapter 4.

The highest proportion of mutants that can be expected to be found in populations of the tubercle bacilli in the absence of selection was shown in Part 1, Chapter 4. These figures condition the size of the inoculum in two out of three methods generally used in testing the susceptibility of the tubercle bacilli. These methods are the *absolute concentration method* and the *resistance-ratio method*; in both, the size of the inoculum should not exceed 100,000 cells. In the third method, known as the *proportion method,* the size of the inoculum is known in every test.

Age of the culture being tested

Once the initial culture reaches maximal growth (at about 6 weeks of incubation), cells begin to die in an unpredictable way. Drug susceptibility tests should be performed with actively growing cultures; if the culture to be tested is too old, it must be subcultured to obtain satisfactory growth for testing. The testing of cultures abandoned on the bench for several months is of doubtful value; in our experience, cultures kept at -70°C, whether on solid medium or frozen in liquid medium, remain satisfactory for over 1 year.

Choice of the medium — phenotypic resistance

The concentration of a drug that inhibits the growth of the tubercle bacilli may vary considerably from medium to medium. In the Lowenstein-Jensen medium the drugs are added before inspissation, and some exhibit low stability to the combination of heat and time required to inspissate the medium; this inconvenience is obviated in agar-base media. Components of the media may interfere with the biological activity of the drug; for example, the egg-yolk phospholipids antagonize streptomycin; D-alanine antagonizes D-cycloserine; methionine antagonizes PAS; polyamines antagonize ethambutol; and PZA is effective only at low pH. These few examples of phenotypic variation indicate that the M.I.C.'s obtained in one type of medium cannot be adopated when using a different medium; in other words, each laboratory must establish the M.I.C.'s applicable to the conditions of testing it elected.

In our laboratory, we prefer the Middlebrook and Cohn 7H-10 medium for use in drug-susceptibility tests.

•Sterilization of the Drugs

The procedure used to sterilize the drugs to be added to the medium must be considered, because the amount of drug that is lost varies with the procedure. Table 2 shows the percent of active drug remaining after sterilization by four different methods.

TABLE 2. Percent of Active Drug Remaining after Autoclaving, Filtering, and Freezing

Drug at 10,000mcg./ml.	Method of sterilization				Stored at -20°C.		
	Autoclave	Seitz	Glass	Millipore	3 mo.	5 mo.	6 mo.
Isoniazid	90	92	100	98	98	98	97
Streptomycin	90	88	95	95	95	92	90
PAS	100	90	100	100	85	80	75
Kanamycin	98	90	96	98	98	98	98
Ethionamide	90	80	90	90	95	88	85
Viomycin	94	95	98	95	98	95	94
Ethambutol	98	80	88	90	90	90	90
Cycloserine	0	98	98	98	85	75	65
Pyrazinamide	100	100	100	100	100	100	100
Rifampin	—	—	—	100	91	86	—

Safety in the Tuberculosis Laboratory

The risk of infection for the laboratory personnel is high unless precautions are taken (*5,46*). Relative to other infections acquired in laboratories handling infectious materials, tuberculosis has been listed fourth, following typhoid fever, brucellosis, and Q fever (*87*).

Because tuberculosis infection is acquired more often by the inhalation of aerosol particles, and one bacillus can start infection, emphasis is placed on the control of aerosols. Most procedures in the laboratory create aerosols, as it is exemplified in Table 3. Aerosols containing tubercle bacilli are also produced in rooms where secretions are collected, either spontaneous sputum, induced sputum, or gastric material (*84*). Therefore, laboratories engaged in tuberculosis work must meet certain operational requirements such as:

1. Existence of an isolation room where specimens are handled.
2. Controlled access to isolation room.
3. Negative air pressure in isolation room.
4. Biological safety hoods and cabinets.
5. Strict disinfection policy.
6. Bulb pipetting required.
7. Special aerosol precautions (centrifuges, blenders, etc.)
8. Air circulation (12 air changes per hour) in isolation room.
9. Air sterilization by ultraviolet light radiation.

Other safety procedures are common to any bacteriology laboratory. Phenol is the most potent disinfectant against the mycobacteria and if used in the isolation room the workers must wear rubber gloves. Because phenol accumulates in the air in the laboratory (*16*), and exposure to its vapors may cause severe reactions, good ventilation where autoclaves are located is recommended.

TABLE 3. Bacteria Recovered by Air Sampling Within 2 Feet of the Site of Common Bacteriological Procedures
[adapted from Wedum, *86*]

Procedure	Colonies obtained per operation
Removing tight cover of standard Waring blender immediately after mixing culture	1
Opening lyophile culture tube	86[1]
Decanting centrifuged fluid into flask	17
Inserting hot loop in culture flask	9
Removing dry cotton plug frm shaken culture flask	5
Pipetting 1 ml of inoculum to poured agar petri plate	3
Pipetting 1 ml of culture into 50 ml of broth	1

[1] Too numerous to count

REFERENCES

1. American Lung Association – Diagnostic Standards and Classification of Tuberculosis, 1973.
1a. Beam, R. E. and Kubica, G. P. (1968) Amer. J. Clin. Pathol. 50:395.
2. Bloch, H. and Noll, H. (1950) J. Exp. Med. 91:197.
3. Bloch, H. (1955) Brit. J. Exp. Pathol. 36:8.
4. Bloch, H. (1956) Adv. Tuberc. Res. 7:149.
5. Bogen, E. (1959) Amer. J. Clin. Pathol. 31:343.
6. Braynen, D. T. and Souger, J. R. (1971) Appl. Microbiol. 22:1166.
7. Calmette, A. (1923) Tubercle bacillus infection and tuberculosis in man and animals. Williams & Wilkins Co., Baltimore, Md., U.S.A., 1923.
8. Canetti, G. et al. Bull Wld. Hlth. Org. 29:565.
9. Canetti, G., Rist, N. and Grosset, J. (1963) Rev. Tuberc. Pneumol. (France) 27:218.
10. Canetti, G. (1972) Bull. Int. Union Against Tub. 47:116.
11. Canetti, G. (1955) The Tubercle bacillus in the pulmonary lesion of man. Springer Publishing Co., Inc., New York, N.Y., 1955.
12. Carvalho, A. (1932) Zeit. Tuberk. 63:305.
13. Castets, M., Rist, N., and Boisvert, H. (1969) Medicine d'Afrique Noir 4:321.
14. Chapman, J. S. (1967) Medical Clinics of North America 51:503.
15. Clancey, J. K. Dodge, O. G., Lunn, H. F. and Odnori, M. L. (1961) Lancet 2:951.
16. Clancey, J. K. (1964) J. Pathol. Bacteriol. 88:175.
17. Cochrane, R. G. and Davey, T. F. (1964) "Leprosy in Theory and Practice," John Wright and Sons, Ltd., Bristol, England, 1964.
18. Corper, H. J. and Uyei, N. (1930) J. Lab. Clin. Med. 15:348.
19. Cruickshank, D. B. (1947) Tubercle 28:97.
19a. Cruickshank, D. B. (1952) Bacteriology of Tuberculosis, in "Modern Practice of Tuberculosis," T. H. Sellors and J. L. Livingston, Eds., Butterworth, London, 1952.
20. Darzins, E. (1958) "The Bacteriology of Tuberculosis," Univ. of Minnesota Press, Minn., 1958.
20a. David, H. L. and Alemquer, M. (1960) Acta Tub. Scand. 39:172,185,194.
21. Davies, R. (1940) Brit. J. Exper. Pathol. 21:243.
22. Dubos, R. and Middlebrook, G. (1948) Amer. Rev. Tuberc. 58:698.
23. Dubos, R. and Middlebrook, G. (1947) Amer. Rev. Tub. Resp. Dis. 56:334.
24. Edwards, P. Q. and Colton, M. L. (1970) "Factors About Tuberculin Testing," Publication of the D.H.E.W., C.D.C., Atlanta, Georgia, U.S.A.
25. Freeze, E., Sklarow, S. and Freeze, E. B. (1968) Mutation Research 5:343.
26. Goldman, D. S. (1969) personal communication.
27. Goren, M. B. (1970) Biochem. Biophys. Acta. 210:116,127.
28. Goren, M. B. (1972) Bacteriol. Rev. 36:33.
29. Goren, M. B., Brokl, O., Das, B. C., and Lederer, E. (1971) Biochem. 10:72.
30. Grunberg, E. (1952) Amer. Assoc. Adv. Science, Minutes of a meeting.
31. Guidelines for the general hospital in the admission and care of tuberculosis patients, (1968) Amer. Rev. Resp. Dis. 99:631.
32. Jensen, K. (1952) Bull. Int. Un. Against Tub. 29:82.
32a. Karassova, V., Weissfeiler, J. and Krasznay, E. (1965) Acta Microbiol. Ac. Sc. Hung. 12:276.
33. Karlson, A. G. and Ikemi, V. (1954) Proc. Mayo Clinic 24:85.
34. Kenney, M., Loechel, A. B. and Lovelock, F. J. (1960) Amer. Rev. Resp. Dis. 82:564.
35. Kestle, D. G. and Kubica, G. P. (1967) Techn. Bull Reg. Med. Technol. 37:347.
36. Koch, R. (1882) Deutsch. Med. Warsch. 16:1029.
37. Krasnow, I. and Wayne, L. G. (1966) Amer. J. Clin. Pathol. 45:352.
38. Kubica, G. P., Dye, W. E., Cohn, M. L. and Middlebrook, G. (1963) Amer. Rev. Resp. Dis. 87:775.
39. Kubica, G P., Kaufmann, A. J. and Dye, W. E. (1964) Amer. Rev. Resp. Dis. 89:284.
40. Kubica, G. (1973) Amer. Rev. Resp. Dis. 107:9.
41. Krasnow, I. and Kidd, G. C. (1965) Amer. J. Clin. Pathol. 44:238.
42. Lester, W. (1970) Clinical Notes in Respiratory Diseases, Vol. 9, No. 2, 1970.

43. Lester, W. (1971) "Treatment of drug-resistant tuberculosis," Year Book Medical Publishers, Inc., Chicago, 1971.
44. Loudon, R. G. and Roberts, R. M. (1968) Amer. Rev. Resp. Dis. 98:297.
45. Long, E. R. (1924) Tubercle 6:128.
46. Long, E. (1951) Amer. J. Pub. Hlth. 41:7.
47. MacCallum, P., Tolhurst, J. C., Buckle, G. and Sisson, H. A. (1948) J. Pathol. Bacteriol. 60:93.
47a. Mackaness, G. B. (1968) Amer. Rev. Resp. Dis. 97:337.
48. Marks, J. and Jenkins, P. A. (1972) Tubercle 53:210.
48a. Marks, J. and Schwabacker, H. (1965) Brit. Med. J. 1:32.
49. Medical Research Council (England). Isoniazid trial: Report No. 4 (1953) Lancet 2:217.
50. Merrill, M. H. (1931) J. Bacteriol. 21:361.
51. Middlebrook, G., Dubos, R. and Pierce, G. (1947) J. Exp. Med. 86:175.
52. Middlebrook, G. (1954) Amer. Rev. Tuberc. 69:471.
53. Middlebrook, G., Cohn, M. L. and Schaefer, W. B. (1954) Amer. Rev. Tuberc. 70:852.
54. Middlebrook, G. and Cohn, M. L. (1958) Amer. J. Pub. Hlth. 48:844.
55. Muir, E. (1948) "Manual of Leprosy," E & S Livingston Ltd., Edinburgh, 1948.
55a. Nicholson, D. P. and Sevier, W. R. (1971) Amer. Rev. Resp. Dis. 104:747.
56. North, C. E. and Park, W. H. (1927) Amer. J. Hyg. 7:147.
57. Novy, F. G. and Soule, M. H. (1925) J. Infect. Dis. 35:168.
58. Oatway, W. H., Jr., and Steenken, W., Jr. (1936) J. Infect. Dis. 59:306.
59. Oliver, J. and Reusser, T. R. (1942) Amer. Rev. Tuberc. 45:450.
60. Petroff, S. A. (1915) Bull. John Hopkins Hosp. 26:276.
61. Proskauer, B. and Beck, M. (1894) Z. Hyg. Infek. 18:128.
62. Pyle, M. (1947) Proc. Mayo Clinic 22:465.
63. Reid, J. S. (1947) Med. J. Aust. 1:427.
64. Reznikov, M. and Robinson, E. (1970) Aust. Vet. J. 46:606.
65. Rich, A. R. (1951) "The pathogenesis of tuberculosis," 2nd Ed., Charles C. Thomas, Springfield, Ill., 1951.
66. Riley, R. L. and O'Grady, F. (1961) "Airborne infection: transmission and control," The MacMillan Company, New York, 1961.
67. Sauton, B. (1912) Compt. Rend. Sc. Soc. Biol. (France) 155:860.
67a. Schaefer, W. B., Wolinsky, E., Jenkins, P. A. and Marks, J. (1973) Amer. Rev. Resp. Dis. 108:1320.
68. Schwabacher, H. (1959) J. Hyg. 57:57.
68a. Selva-Sutter, E., Silcox, V. A. and David, H. L. (1975) Appl. Microbiol. (submitted).
69. Shepard, C. C. (1962) Int. J. Leprosy 30:291.
70. Shepard, C. C. and Chang, Y. T. (1962) Proc. Soc. Exptl. Biol. Med. 109:636.
71. Shepard, C. C. and McRae, D. H. (1965) J. Bacteriol. 89:365.
72. Shepard, C. C. (1967) Int. J. Leprosy 35:429.
73. Silcox, V. A. and David, H. L. (1971) Appl. Microbiol. 21:327.
74. Smith, R. C. (1942) Amer. Rev. Resp. Dis. 45:334.
75. Smith, T. (1896) Trans. Ass. Amer. Phys. 11:75.
76. Smith, T. (1898) J. Expt. Med. 3:451.
76a. Smith, T. (1910) J. Med. Res. 23:185.
77. Smithwick, R. W. and David, H. L. (1971) Tubercle 52:226.
78. Spendlove, G. A., Cummings, M. M. and Patnode, R. A. (1949) Amer. Rev. Tuberc. 68:548.
78a. Steenken, W., Jr. and Wolinsky, E. (1953) Amer. Rev. Ruberc. 60:628.
79. Steiner, M. Zommerman, R., Park, B. H. Shiralis, S. R. and Schmidt, H. (1968) Amer. Rev. Resp. Dis. 98:201.
80. Steiner, P. and Portugaleza, C. (1973) Amer. Rev. Resp. Dis. 107:22.
81. Stonebrink, B. (1958) Acta. Tuberc. Scand. 35: Fasc. 1.
82. The Tuberculin Skin Test — A Statement by the Committee on Diagnostic Skin Test (1971) Amer. Rev. Resp. Dis. 104:769.
83. Uganda Buruli Group (1969) Lancet 1:111.
84. Vandiviere, H. M., Smith, C. E. and Sunkes, E. J. (1953) Amer. Rev. Tuberc. 67:878.
85. Wayne, L. G. and Krasnow, I. (1966) Amer. J. Clin. Pathol. 45:769.
86. Wedum, A. (1964) Pub. Hlth. Rep. 79:619.

87. Wedum, A. and Kruse, R. H. (1969) "Assessment of risk of human infection in the microbiological laboratory," Department of the Army, Fort Detrick, Maryland, U.S. Printing Office, AD 693:258.

88. Wells, W. F. (1947) Amer. J. Hyg. 47:1.

89. Wells, W. F. (1947) Amer. J. Hyg. 47:11.

90. WHO Technical Report Series (1953) 71:25.

91. W.H.O. Expert Committee on Tuberculosis, IX Report, WHO, Geneva, 1974.

92. Widelock, D., Peizer, L. R. and Klein, S. (1953) Amer. Rev. Tuberc. 68:290.

93. Willis, H. S. and Cummings, M. M. (1952) "Diagnostic and Experimental Methods in Tuberculosis," Charles C. Thomas Publishers, Springfield, Ill., 1952.

94. Yegian, D. and Budd, V. (1955) Amer. Rev. Tuberc. 72:539.

☆U.S. GOVERNMENT PRINTING OFFICE: 1975 648-193

DHEW Publication No. (CDC) 76-8316